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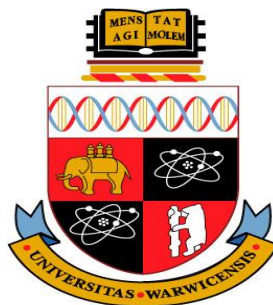
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Role of microbial adaptation in the biodegradation of chemical pollutants: extrapolation from laboratory to rivers

by

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Doctor of Philosophy



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Table of contents

Table of contents	i
List of tables.....	viii
List of figures.....	x
Abbreviations	xii
Acknowledgements	xvii
Declaration.....	xviii
Abstract.....	xix
Chapter 1	1
Introduction.....	1
1.1. Down-The-Drain chemicals	1
1.2. Pathway for entry of DTD into the environment including geographical differences	4
1.3. Ecotoxicological implications of chemical release into the environment.....	8
1.4. Regulatory drivers for chemical management	10
1.4.1. REACH and PBT assessment	10
1.4.2. European Union and REACH.....	13
1.5. Methods for determining biodegradation rates	14
1.5.1. Predictive modelling	14
1.5.2. OECD tests.....	15
1.5.2.1. Role of biodegradation testing	15
1.5.2.2. Historical aspects and principal design of OECD tests.....	15
1.5.2.3. Overview of current tests	19
1.5.2.3. Overview of current tests	19
1.5.2.3.1. Ready biodegradability tests (screening tests)	19
1.5.2.3.2. Inherent (potential) biodegradability tests	20
1.5.2.3.3. Simulation tests	20
1.6. Environmental realism of tests.....	23
1.6.1. Effect of environmental factors on biodegradability assessment.....	23
1.6.2. Water chemistry	27
1.6.3. Inoculum	28
1.6.3.1. Biofilm as inoculum.....	31
1.6.4. Bioavailability.....	33
1.6.5. Test substance concentration	34
1.6.6. Kinetics	36

1.7. Microbial ecology in biodegradation testing.....	37
1.8. Future perspectives	39
1.8.1. Metagenomics	41
1.8.2. Metatranscriptomics.....	41
1.8.3. Metaproteomics.....	42
1.8.4. Systems biology approach to biodegradation	42
1.8.5. Advantages and disadvantages of high throughput methods	43
1.9. <i>Para</i> -nitrophenol (PNP).....	44
1.9.1. PNP in the environment	44
1.9.2. Application and properties of <i>para</i> -nitrophenol	45
1.9.3. <i>Para</i> -nitrophenol as a model compound	46
1.9.4. Biodegradation pathways and functional genes	47
1.10. Research area	49
1.10.1. River Dene	49
1.10.2. Wastewater Treatment Plants (WWTPs)	52
1.10.3. Flood events	53
1.11. Aims and objectives of the project.....	55
Chapter 2	57
The biodegradation of <i>para</i>-nitrophenol under light and dark conditions: a functional gene approach.....	57
2.1. Introduction.....	57
2.2. Objectives	61
2.3. Materials and methods	62
2.3.1. Sample collection.....	62
2.3.2. Experimental set up.....	62
2.3.3. Measurement of PNP biodegradation	65
2.3.4. Flow cytometry	65
2.3.5. Determination of total viable count (colony forming units)	66
2.3.5.1. Inoculum size	66
2.3.5.2. Isolation of PNP-degrading bacteria	66
2.3.6. Colony PCR	67
2.3.7. ¹⁴ C PNP biodegradation assay with bacterial isolates.....	68
2.3.7.1. Experimental set up.....	69
2.3.7.2. Measurement of PNP and ¹⁴ C PNP biodegradation	69
2.3.8. Effect of light and pH on PNP biodegradation	70
2.3.9. Terminal Restriction Fragment Length Polymorphism (TRFLP).....	70

2.3.10. Primer design for <i>pnpA</i> , <i>npdA2</i> and <i>mar</i>	71
2.3.11. Optimisation of PCR conditions for amplification of PNP biodegradation genes.....	73
2.3.12. Detection of <i>pnpA</i> , <i>npdA2</i> and <i>mar</i>	76
2.3.13. Clone libraries of <i>pnpA</i> and <i>mar</i>	76
2.3.14. Phylogenetic analysis of sequences of functional genes.....	77
2.3.15. Quantitative PCR of <i>pnpA</i> and <i>mar</i> genes.....	78
2.3.16. 454 sequencing for <i>pnpA</i> and <i>mar</i> gene.....	80
2.3.17. Analysis of 454 sequence data.....	81
2.3.18. Statistical analyses.....	82
2.3.18.1. Statistical analysis of PNP biodegradation and QPCR data.....	82
2.3.18.2. Statistical analysis of bacterial community diversity using TRFLP profiles ..	82
2.3.18.3. Statistical analysis of the PNP degrading communities using <i>pnpA</i> and <i>mar</i> sequences	83
2.4. Results.....	85
2.4.1. The biodegradation of PNP.....	85
2.4.2. Inoculum size	86
2.4.3. Flow cytometry measurements	86
2.4.4. Isolation and identification of PNP degraders	87
2.4.5. pH measurement	90
2.4.6. Direct effect of light and pH on the biodegradation of PNP.....	91
2.4.7. Bacterial community profile using TRFLP.....	92
2.4.8. QPCR for <i>pnpA</i> and <i>mar</i>	95
2.4.9. Functional marker analysis	97
2.4.9.1. Sequencing and clone library for <i>pnpA</i>	97
2.4.9.2. Sequencing and clone library for <i>mar</i>	99
2.4.10. 454 sequencing for PNP functional genes	102
2.4.10.1. 454 for <i>pnpA</i>	102
2.4.10.2. 454 for <i>mar</i>	104
2.5. Discussion.....	108
2.5.1. Effect of light on the biodegradation of PNP and river water bacterial community structure	108
2.5.2. Effect of PNP concentration on the biodegradation of PNP and river water bacterial community structure	109
2.5.3. Identification of PNP degrading bacterial populations	111
2.5.4. PNP biodegradation pathway.....	113
2.6. Conclusions.....	114

Chapter 3	116
The influence of microbial inoculum and water chemistry on the biodegradation of <i>para</i>-nitrophenol.....	116
3.1. Introduction.....	116
3.2. Objectives	120
3.3. Materials and methods	121
3.3.1. Sample collection and preparation	121
3.3.2. Experimental set up.....	123
3.3.3. Measurement of PNP biodegradation	123
3.3.4. Measurement of ammonium, nitrite and nitrate	123
3.3.5. Determination of total viable count (colony forming units)	124
3.3.5.1. Inoculum size	124
3.3.5.2. Isolation of specific degraders and phylogenetic tree for 16S rRNA gene	124
3.3.6. Colony PCR	125
3.3.7. Terminal Restriction Fragment Length Polymorphism (TRFLP).....	125
3.3.8. Detection of PNP functional genes	125
3.3.9. Quantitative PCR of <i>pnpA</i> and <i>mar</i> genes	125
3.3.10. Statistical analyses	125
3.4. Results.....	126
3.4.1. The biodegradation of PNP by different inocula	126
3.4.2. Water and sediment chemistry	128
3.4.3. Inoculum size and number of PNP degrading bacteria.....	130
3.4.3.1. Bacterial isolates degrading PNP	132
3.4.4. TRFLP profiling of bacterial communities from environmental samples before and after PNP biodegradation	135
3.4.5. Quantification of <i>pnpA</i> and <i>mar</i> functional marker copy number by QPCR.....	141
3.5. Discussion.....	143
3.5.1. Effect of inoculum material and location on the biodegradation of PNP and microbial community structure in river water and sediment	143
3.5.2. Effect of effluent discharge on the biodegradation of PNP and microbial community structure in river water and sediment.....	145
3.6. Conclusions.....	149
Chapter 4	151
Effect of inoculum density and diversity on <i>para</i>-nitrophenol biodegradation.....	151
4.1. Introduction.....	151
4.2. Objectives	154
4.3. Materials and methods	155

4.3.1. River Dene biofilms	155
4.3.2. The biodegradation of PNP by river biofilms	156
4.3.3. Biofilm biomass	156
4.3.3.1. Total carbohydrates extraction	156
4.3.3.2. Bacterial viable count	157
4.3.3.3. DNA extraction from biofilms	157
4.3.4. Microbial ecology methods	158
4.3.5. Seasonal conditions	158
4.3.5.1. Light and temperature	158
4.3.5.2. Water quality	158
4.3.5.3. Flow rate	159
4.3.6. Statistical analyses	159
4.3.6.1. Analysis of biodegradation and QPCR data	159
4.3.6.2. Analysis of TRFLP profiles	160
4.3.6.3. Analyses for core and satellite species from TRFLP profiles	160
4.3.6.3.1. Analysis of the local community	160
4.3.6.3.2. Analysis of the metacommunity	161
4.4. Results	162
4.4.1. The biodegradation of PNP	162
4.4.2. Biofilm biomass	165
4.4.3. Seasonal variation in physicochemical parameters	166
4.4.3.1. Water quality	166
4.4.3.2. Flow data	166
4.4.4. Biofilm and river water bacterial TRFLP profiles	170
4.4.4.1. NMDS analysis of TRFLP data	170
4.4.4.2. Total number of Terminal Restriction Fragments (TRFs)	172
4.4.5. Abundance of TRF 245 nt	174
4.4.6. Core and satellite taxa	177
4.4.6.1. Biofilm local community structure and abundance of <i>Pseudomonas syringae</i>	177
4.4.6.2. Biofilm metacommunity structure, distribution and persistence of <i>Pseudomonas syringae</i>	178
4.4.7. Factors affecting biofilm bacterial community structure	181
4.4.8. Quantitative PCR analysis of functional genes	183
4.4.8.1. Copy number of <i>pnpA</i>	183
4.4.8.2. Copy number of <i>mar</i>	183

4.4.8.3. Copy number of 16S rRNA gene	184
4.5. Discussion.....	188
4.5.1. Effect of sampling date on the biodegradation of PNP and microbial community composition.....	188
4.5.2. Effect of sampling site location on the biodegradation of PNP and microbial community composition.....	190
4.5.3. Effect of inoculum material on the biodegradation of PNP and microbial community composition.....	191
4.6. Conclusions.....	193
Chapter 5	196
Threshold PNP concentration for the biodegradation of PNP	196
5.1. Introduction.....	196
5.2. Objectives	200
5.3. Materials and methods	201
5.3.1. Inoculum material	201
5.3.2. Experimental set up.....	201
5.3.3. ¹⁴ C-labelled PNP and PNP-radiation and chemical concentration.....	202
5.3.4. Monitoring the biodegradation of parent ¹⁴ C labelled PNP and PNP	202
5.3.4.1. Radio-HPLC	202
5.3.4.2. Scintillation counts.....	203
5.3.5. Microbial ecology methods.....	203
5.3.6. Statistical analyses	203
5.4. Results.....	204
5.4.1. The effect of PNP concentration on the biodegradation rate of PNP.....	204
5.4.2. Biofilm biomass	208
5.4.3. Effluent biofilm bacterial TRFLP profiles.....	208
5.4.3.1. All Terminal Restriction Fragments.....	208
5.4.3.2. TRF 245 nt	210
5.4.4. QPCR for PNP functional genes and 16S rRNA gene.....	212
5.5. Discussion.....	214
5.5.1. The effect of PNP concentration on the PNP biodegradation rate and threshold PNP concentration.....	214
5.5.2. Effect of PNP concentration on effluent biofilm TRFLP profiles, abundance of PNP-degraders and potential for PNP biodegradation.....	216
5.6. Conclusions.....	218
Chapter 6	220
General discussion	220

6.1. Variability of microbial inocula.....	220
6.2. Inoculum characterization.....	224
6.3. Test design	226
6.4. Methods to characterize biodegradation potential	227
6.5. Future recommendations.....	228
6.6. Future work.....	230
6.6.1. Inoculum variability.....	230
6.6.2. Functional marker approach.....	230
6.6.3. The use of the biodegradation studies and decision making.....	231
6.6.3.1. Persistence of chemicals in the environment	231
6.6.3.2. Modelling.....	232
References.....	234
Appendices.....	267

List of tables

Table 1.1: Examples of HPCs and their global usage in 2011.....	3
Table 1.2: Reference chemicals and their classification into BINs.....	17
Table 1.3: Environmental variables, temperature and light, affecting the biodegradation of chemicals.....	25
Table 1.4: Environmental variables, oxygen and bed form, affecting the biodegradation of chemicals.....	26
Table 1.5: Application and potential of high-throughput technologies in biodegradation studies.	40
Table 1.6: Physico-chemical properties of <i>para</i> -nitrophenol.	46
Table 1.7: Water quality data for River Dene.....	52
Table 1.8: Wastewater Treatment Plants within River Dene catchment.....	54
Table 2.1: Chemical compounds used in this study.....	64
Table 2.2: Strains used in this study.	64
Table 2.3: Primers used in this study	68
Table 2.4: The DT-values for PNP biodegradation in river water under dark conditions.	86
Table 2.5: Properties of bacterial isolates.	88
Table 2.6: Changes in river water pH after incubation period of eight and 36 days in the dark and light, respectively.	90
Table 2.7: Relative abundance of TRF 245 nt in bacterial TRFLP profiles of river water....	94
Table 2.8: Diversity of <i>mar</i> clones from T ₀ and PNP degrading river water based on <i>mar</i> neighbour-joining tree.....	101
Table 2.9: Identity and relative abundance of <i>pnpA</i> gene sequences in picked OTUs from T ₀ river water and water degrading high PNP concentration in the dark (HD).	103
Table 2.10: Identity and relative abundance of <i>mar</i> gene sequences in picked OTUs from T ₀ river water and from river water degrading PNP at high concentration in the dark (HD)...	106
Table 3.1: Statistical analysis for PNP biodegradation in different treatments.	128
Table 3.2: Chemistry of river water and sediment extracts.	129
Table 3.3: Number of cultivable bacteria and average number of PNP degraders in environmental samples.....	131
Table 3.4: PNP degrading bacterial isolates from river water and sediment extracts after complete PNP biodegradation.....	134
Table 3.5: ANOVA for average number of TRFs present in bacterial TRFLP profiles.	138
Table 3.6: ANOVA for relative abundance of TRF 245 nt in bacterial TRFLP profiles....	140
Table 4.1: DT-values calculated for PNP biodegradation with biofilms and river water. ...	164
Table 4.2: Inoculum size – biofilm biomass determined based on the DNA concentration, EPS concentration, and number of cultivable bacteria.	165
Table 4.3: River Dene water level, flow rate and water discharge.	169
Table 4.4: ANOVA for total number of TRFs present in biofilm and river water TRFLP profiles.	173
Table 4.5: Relative abundance of TRF 245 nt in biofilm and river water TRFLP profiles.	176
Table 4.6: ANOVA test results comparing local biofilm communities from different sampling dates (November, February, May) sampling locations (upstream, effluent, downstream) and before (T ₀) and after complete PNP biodegradation (T _{end}).....	178

Table 4.7: Comparison of slopes of metacommunity rank-abundance plots with core and satellite taxa.	181
Table 5.1: Concentrations of PNP measured in the environment.	197
Table 5.2: Comparison of DT-values, length of lag phase and maximum PNP degradation rate for PNP biodegradation at different PNP concentrations with biofilms.	206
Table 5.3: Comparison of DT-values, length of lag phase and maximum PNP degradation rate for PNP biodegradation at different PNP concentrations with <i>Pseudomonas syringae</i> AKHD2.....	206
Table 5.4: Trapped $^{14}\text{CO}_2$ during the biodegradation of ^{14}C PNP with biofilms and <i>Pseudomonas syringae</i> AKHD2.	208
Table 5.5. Inoculum size – biofilm biomass determined based on the DNA concentration, EPS concentration, and number of cultivable bacteria.	208
Table 5.6: ANOVA for total number of TRFs present in effluent biofilm TRFLP profiles before and after PNP biodegradation at different PNP concentrations.	210
Table 5.7: Relative abundance of TRF 245 nt in effluent biofilm TRFLP profiles before and after PNP biodegradation.	211
Table 6.1: Inoculum size in different biodegradation experiments.....	223

List of figures

Figure 1.1: Pathways of chemical entry into the environment.....	5
Figure 1.2: Relationship between screening and higher tier biodegradability tests and the bins.....	18
Figure 1.3: Relationship of an impact zone caused by the direct discharge of organic wastes, as described by increased BOD, NH ₃ and decreased DO, with biotic integrity.....	28
Figure 1.4: Schematic of an approach to study effects of environmental realism on biodegradation.....	44
Figure 1.5: <i>Para</i> -nitrophenol.	45
Figure 1.6: Pathways for PNP degradation in bacteria.	48
Figure 1.7: Involvement of maleylacetate reductase in bacterial catabolic pathways of aromatic compound degradation.....	49
Figure 1.8: River Dene catchment with local Wastewater Treatment Plants.....	51
Figure 1.9: River Dene catchment with Wastewater Treatment Plants.	53
Figure 2.1: River water incubated under light and dark conditions with PNP.....	63
Figure 2.2: Phylogenetic relationship of the nitroarene monooxygenases and their homologues.	72
Figure 2.3: Optimisation of PCR reaction conditions from newly designed primers for PNP functional genes involved in PNP biodegradation.	75
Figure 2.4: The biodegradation of PNP under light and dark conditions.	85
Figure 2.5: The biodegradation of PNP with bacterial isolates.	89
Figure 2.6: The biodegradation of PNP under a- light and b- dark conditions in mineral medium with low pH 7.5 and high pH 9.8.....	91
Figure 2.7: a- Non-Metric Multidimensional Scaling (NMDS) plot of TRFLP bacterial profiles, b- dendrogram showing similarities between TRFLP profiles and Bray-Curtis distance for the data set derived from amplification with bacterial primers 63F and 1087R-VIC fluorescently labelled, and digested with <i>Msp</i> I and <i>Hha</i> I. P.....	93
Figure 2.8: A 2D bubble plot presenting the amount of TRF 245 nt (TRF identified as <i>Pseudomonas syringae</i>) in environmental samples using Bray-Curtis similarity.....	94
Figure 2.9: QPCR for functional genes and 16S rRNA gene.	96
Figure 2.10: Neighbour-joining tree showing the diversity of <i>pnpA</i> gene sequences obtained from cloning and 454 sequencing.	98
Figure 2.11: Neighbour-joining tree showing diversity of <i>mar</i> gene sequences obtained from cloning and 454 sequencing.....	100
Figure 2.12: The relative abundance of <i>pnpA</i> sequences as % of total <i>pnpA</i> sequences forming OTUs from T ₀ and river water degrading high PNP concentration in the dark (HD)..	103
Figure 2.13: The relative abundance of <i>mar</i> sequences as % of total <i>mar</i> sequences forming OTUs from T ₀ and river water degrading high PNP concentration (HD).....	105
Figure 3.1: River Dene and location of sampling points.	122
Figure 3.2: The biodegradation of PNP with different inocula.....	127
Figure 3.3: A neighbour-joining phylogenetic tree of 16S rRNA gene from PNP degrading isolates.	133

Figure 3.4: Non-metric multidimensional scaling (NMDS) plot of bacterial TRFLP profiles, using Bray-Curtis similarity for the data set derived from amplification with bacterial primers 63F and 1087R- VIC fluorescently labelled and digested with <i>MspI</i> and <i>HhaI</i>	136
Figure 3.5: A 2D bubble plot presenting the amount of TRF 245 nt (TRF identified as <i>Pseudomonas syringae</i>) using Bray-Curtis similarity.....	139
Figure 3.6: QPCR for functional genes and 16S rRNA gene.	142
Figure 4.1: Biofilm trap consisting of brick with attached glass slides.	155
Figure 4.2: The biodegradation of PNP with biofilms and river water.....	163
Figure 4.3: Measurements for COD, ammonia, nitrite and nitrate for river water at upstream, effluent and downstream site.	167
Figure 4.4: Measurements for DOC, temperature, pH and light at upstream, effluent and downstream site.	168
Figure 4.5: Non-metric multidimensional scaling (NMDS) for bacterial TRFLP profiles, using Bray-Curtis similarity for the data set derived from amplification with bacterial primers 63F and 1087R- VIC fluorescently labelled and digested with <i>MspI</i> and <i>HhaI</i>	171
Figure 4.6: Total number of TRFs present in bacterial profiles from river water and biofilms collected in November 2011, February and May 2012.....	172
Figure 4.7: A 2D bubble plot presenting the amount of TRF 245 nt (TRF identified as <i>Pseudomonas syringae</i>) using Bray-Curtis similarity.....	175
Figure 4.8: Rank-abundance plots with partitioning into core and satellite species.	180
Figure 4.9: Non-metric-multidimensional scaling plots showing environmental factors affecting biofilm bacterial community structure at T ₀	182
Figure 4.10: Copy number of <i>pnpA</i> gene.....	185
Figure 4.11: Copy number of <i>mar</i> gene.....	186
Figure 4.12: Copy number of 16S rRNA gene.	187
Figure 5.1: The biodegradation of ¹⁴ C PNP at different PNP concentrations with effluent biofilm.....	205
Figure 5.2: Release of ¹⁴ CO ₂ during the biodegradation of ¹⁴ C PNP and different PNP concentrations.	207
Figure 5.3: Analysis of TRFLP biofilm community profiles.....	209
Figure 5.4: A 2D bubble plot presenting the amount of TRF 245 nt (TRF identified as <i>Pseudomonas syringae</i>) in environmental samples using Bray-Curtis similarity.....	211
Figure 5.5: Effect of PNP concentration on the copy number of PNP functional genes and 16S rRNA gene.	213
Figure 6.1: New approaches for the biodegradation potential assessment prior to biodegradation testing and improved risk assessment for chemicals.....	222

Abbreviations

%	Percentage
% ID	Percentage of identity
°C	Degree Celsius
¹⁴ C	Radioactive carbon-14
¹⁴ CO ₂	Radioactive ¹⁴ C carbon dioxide
2,4-D	2,4-dichlorophenoxyacetic acid
2,4-DNP	2,4-dinitrophenol
2-ANP	2-amino-4-nitrophenol
2D	Two dimensional
4-NC	4-nitrocatechol
<i>alk</i>	Alkane-catabolic gene
AISE	International Association for Soaps, Detergents and Maintenance Products
ANOSIM	Analysis of similarities
ANOVA	Analysis of variance
ARB	A software environment for sequence data
ASL	Activated sludge treatment plant
ASP	Activated Sludge Processes
ASPT	Average score per taxon
atm	Atmosphere
ATSDR	Agency for Toxic Substances and Disease Registry
<i>b</i>	Slope
BLAST	Basic Local Alignment Search Tool
BOD	Biological oxygen demand
bp	Base pair
Bq	Becquerel
BRW	Brewery
BTEX	Benzene, toluene, ethylbenzene and xylenes
C ₆ H ₅ NO ₃	<i>para</i> -nitrophenol
cfu	Colony forming unit
cm	Centimetre
cm ²	Centimetre squared
CO ₂	Carbon dioxide
COD	Chemical oxygen demand
COM	Cow manure
CSA	Chemical safety assessment
DMSP	Dimethylsulfoniopropionate
DNA	Deoxyribonucleic acid
DO	Dissolved oxygen
DOC	Dissolved organic carbon

DSD	Dangerous Substances Directive
DSMZ	German Collection of Microorganisms and Cell Cultures
DT ₁₅	Time to 15% degradation of chemical
DT ₅₀	Time to 50% degradation of chemical
DT ₇₅	Time to 75% degradation of chemical
DT ₉₀	Time to 90% degradation of chemical
DTD	Down-the-drain chemicals
DWI	Drinking Water Inspectorate
E1	Estrone
E2	17β-estradiol
ECETOC	European Centre for Ecotoxicology and Toxicology of Chemicals
ECHA	European Chemicals Agency
ECOSOL	European Council on Studies in LAS and LAB
EDCs	Endocrine disrupting chemicals
EMPA	The Swiss Federal Laboratories for Materials Science and Technology
EPA	Environmental Protection Agency
EPS	Extracellular polysaccharides
ESIS	European Chemical Substances Information System
EU	European Union
F	Degrees Fahrenheit
g	Gram
HERA	Human and Environmental Risk Assessment
<i>HhaI</i>	<i>Haemophilus haemolyticus</i> type I
HPC	Home and personal care products
HPLC	High performance liquid chromatography
HQ	Hydroquinone
HSE	Health and Safety Executive
IECSC	Inventory of Existing Chemical Substances of China
IMG	Integrated Microbial Genome and Metagenome
IND	Index of Multivariate Dispersion
K	Kelvin
kg	Kilogram
KH ₂ PO ₄	Monopotassium phosphate
K _{oc}	Partition coefficient
K _{ow}	Octanol-water partition coefficient
L	Litre
LAB	Linear alkylbenzene
LAE	Linear alcohol ethoxylates
LAS	Linear alkylbenzene sulfonates
LB agar	Luria-Bertani agar
LRTAP	Long-Range Transboundary Air Pollution
LSD	Least significant difference
lux	Luminous flux

m	Meter
m ³	Cubic meter
<i>mar</i>	Maleylacetate reductase gene
MAR	Maleyacetate reductase protein
mg	Milligram
MgSO ₄	Magnesium sulphate
min	Minute
MITI	Japanese Ministry of International Trade and Industry
mL	Millilitre
mm	Millimetre
mM	Millimolar
mn	Million
MRFLP	Macro-Restriction Fragment Length Polymorphism
mRNA	Messenger ribonucleic acid
<i>MspI</i>	<i>Moraxella</i> sp. type I
MTBE	Methyl- <i>tert</i> -butyl ether
MUSCLE	Multiple Sequence Comparison by log-Expectation
Na ₂ HPO ₄	Sodium phosphate dibasic
NACEC-SMOC	North America Commision for Environmental Cooperation, Sound Management of Chemicals
NADH	Reduced nicotinamide adenine dinucleotide
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NaOH	Sodium hydroxide
NCBI	National Centre for Biotechnology Information
NED	N-(1-naphtyl)-ethylene diamine
NERRS	National Estuarine Research Reserve System
ng	Nanogram
NH ₃	Ammonia
NH ₄ ⁺	Ammonia ion
NH ₄ Cl	Ammonium chloride
nm	Nanometer
NMDS	Non-Metric Multidimensional Scaling
NMOR	N-nitrosomorpholine
NO ₂ ⁻	Nitrite ion
NO ₃ ⁻	Nitrate ion
NP	Nonylphenol
<i>npdA2</i>	PNP 2-monooxygenase gene
NpdA2	PNP 2-monooxygenase protein
NPEOs	Nonylphenol ethoxylates
nt	Nucleotides
OECD	Organisation for Economic Cooperation and Development
OTUs	Operational Taxonomic Units
PAH	Polycyclic aromatic hydrocarbon
pBQ	<i>para</i> -benzoquinone

PBT	Persistent, bioaccumulative and toxic
PCDD/Fs	Polychlorinated dibenzo- <i>p</i> -dioxins and dibenzofurans
PCR	Polymerase chain reaction
PEC	Predicted environmental concentration
pKa	Dissociation constant
PLFA	Phospholipid Fatty Acid
PMNs	Premanufacture notice chemicals
PNEC	Predicted non-effect environmental concentration
PNP	<i>para</i> -nitrophenol
<i>pnpA</i>	PNP 4-monooxygenase gene
PnpA	PNP 4-monooxygenase
POPs	Persistent organic pollutants
PPCPs	Pharmaceuticals and personal care products
QPCR	Quantitative PCR
QSARs	Quantitative structure-activity relationships
r^2	Coefficient of determination
RBT	Ready biodegradability tests
REACH	Registration, Evaluation and Authorisation of Chemicals
RDP Classifier	Ribosomal Database Project Classifier
RNA	Ribonucleic acid
RPM	Rotation per minute
rRNA	Ribosomal ribonucleic acid
RTL	Research and Testing Laboratory
S.E.M.	Standard Error of the Mean
SARs	Quantitative structure relationships
SBR	Sequencing batch reactor
SCAS	Semi-continuous activated sludge
SCEP	Semi-continuous pre-exposure procedure
sec	Seconds
T	Testosterone
T ₀	Time zero
TA2	Tertiary A2 treatment
TC	Total carbon
TCC	Triclocarban
TCS	Triclosan
T _{end}	Time after complete PNP biodegradation
TGD	Technical Guidance Document
ThCO ₂	Theoretical carbon dioxide
ThOD	Theoretical oxygen demand
TIC	Total inorganic carbon
T _m	Melting temperature
TOC	Total organic carbon
TPH	Total Petroleum Hydrocarbon
TRF	Terminal Restriction Fragment

TRFLP	Terminal Restriction Fragment Length Polymorphism
UASB	Upflow anaerobic sludge blanket
UNECE	United Nations Economic Commission for Europe
UNEP	United Nations Environment Programme
vPvB	Very persistent, very bioaccumulative
WWTP	Wastewater Treatment Plant
μg	Microgram
μL	Microlitre
μm	Micrometer
μM	Micromolar
χ^2	Index of dispersion

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Declaration

I declare that the work presented in this thesis was conducted by me under the direct supervision of Doctor Gary Bending and co-supervision of Doctor Hendrik Schäfer, Doctor Oliver Price, Doctor Roger van Egmond and Doctor Chris Finnegan, with the exception of those instances where the contribution of others has been specifically acknowledged. None of the work presented has been previously submitted for any other degree.

Agnieszka Kowalczyk

Abstract

The Organisation for Economic Cooperation and Development (OECD) established standardized tests to predict the chemical behaviour in the environment. Difficulties exist in the extrapolation of data from laboratory OECD tests into the environment, and prediction of possible scenarios regarding environmental pollution is not accurate. The current project aimed to increase the realism of OECD tests and to investigate the microbial populations involved in the biodegradation of *para*-nitrophenol (PNP). River water, sediment and biofilms were sampled from a stretch of River Dene with an effluent discharge point (Wellesbourne WWTP), and were used as microbial inocula in biodegradation studies. The effect of light, PNP concentration and inoculum preparation on PNP biodegradation was determined. Culture dependent techniques were used for isolation of PNP-degrading bacteria while culture independent techniques; including 16S rRNA Terminal Restriction Fragment Length Polymorphism, QPCR, and high throughput sequencing targeting the PNP functional genes (*pnpA* and *mar*), enabled detection and characterization of PNP-degrading bacteria. Light incubation lead to increased river water pH which inhibited PNP degradation. A threshold PNP concentration was determined around 42 µg/L. Application of biofilm inocula improved the reproducibility of PNP biodegradation at a concentration of 2 mg/L, and increased the amount of microbial biomass in test systems. *Pseudomonas syringae* was identified as a key PNP-degrader. Additional groups of PNP-degrading bacteria were detected based on the analysis of *pnpA* and *mar* functional markers. It was shown that location of sampling site for inoculum collection had no impact on biodegradation test outcome but variation of microbial inocula between sampling dates may affect the biodegradation of PNP. Core and satellite taxa analysis demonstrated that ‘biodegradation lottery’ is not the major process which determines the successful chemical biodegradation, and that probably microbial interactions within inoculum affect proliferation of PNP-degraders, and therefore impact on test results. This project revealed the lack of consideration of microbial inocula in the OECD biodegradation test guidelines. Further experimental work was suggested to expand current studies to different chemicals and rivers, and to develop more predictable approaches for better chemical risk management by Industry and Regulatory bodies.

Chapter 1

Introduction

1.1. Down-The-Drain chemicals

Over the past decades increasing concerns have been raised about the potential adverse human and ecological health effects resulting from the production, use, and disposal of chemicals used in industry, agriculture, medical treatment, and common household products (Lapertot and Pulgarin, 2006). A key group of chemicals which are of the concern are the so-called “down-the-drain” chemicals. “Down-the-drain” chemicals (DTD), also referred to as “daily use chemicals”, include ingredients used in domestic consumer products (e.g. detergent ingredients such as surfactants, solvents, dyes, perfumes and bleaching agents) and pharmaceuticals, which may be disposed of with household wastewater (Whelan *et al.*, 2011). The major source of DTD chemicals are home and personal care products (HPC). Chemicals that are components of HPC products number in the thousands, and are contained in skin care products, dental care products, soaps, hair care products, laundry products and household products (Richardson *et al.*, 2005; Brausch and Rand, 2011). The greatest use of HPC in Europe is for laundry products, followed by dishwashing detergents and surface care products (i.e. multipurpose, bathroom, kitchen and floor cleaners), with 8837, 2240 and 1749 mL/capita/year, respectively in 2010 (Price *et al.*, 2010). Usage of domestic and personal care products has been related to gross domestic product, so that high GDP thresholds (> 10,000 US \$) are required before there is substantial use of deodorants and machine dishwasher products and low GDP (< 500 US \$) for bar soap (Hodges *et al.*, 2012). According to Euromonitor International (2012), a company carrying out strategy research for consumer markets, the global use of HPCs from 2006–2011 varied regionally with noticeable difference between developed countries (North America, Western Europe) and developing countries (Africa, Asia) (**Table 1.1**). Price *et al.* (2010) reported the highest estimated use of HPC products in two regions in Europe, first Southern England and second a

transboundary area in Europe containing Belgium, the Netherlands, and Germany. Hodges *et al.* (2012) reported that HPC product usage also varies across China, and is typically higher in East and South China, relative to the Northwest.

Among personal care products, bar soaps, body wash/shower gel, conditioners, standard shampoos, general-purpose body care products, toothpastes and dental rinses are mainly used. In terms of household cleaning products, the most popular are standard and concentrated powder detergents, household antiseptics/detergents, bar detergents, and bleach. Vast majority of HPCs are laundry products. They are used in high tonnages around the world. A.I.S.E., the International Association for Soaps, Detergents and Maintenance Products, reported that in 2011, in Europe the total household value of HPCs was estimated at 27.7 billion Euros and is showing annual growth of approximately 1%. Laundry care (48%) and surface care products (21%) contributed largely to the household sector, followed by dishwashing (15%), maintenance products (i.e. products for wood, leather and metal surfaces, insecticides, disinfectants, air fresheners) (14%) and bleaches (2%) (AISE, 2012). The production of surfactants, largely reflecting soap (50%); with linear alkylbenzene sulfonates (LAS) and derivatives of alcohols, is also important (Sanz *et al.*, 2006). The world production capacity in the year 2005 just for linear alkyl benzene (LAB) alone, the precursor of LAS, was estimated to be more than 3 million tons. In 2005, the estimated total amount of LAB used in Europe was around 600 kilotons/year. A survey completed by the European Council on Studies on LAB and LAS (ECOSOL) estimated a total LAS consumption tonnage of about 430 kilotons for the year 2005, with the proportion used in household applications being about 350 kilotons (HERA, 2009). The total market for surfactants in Western Europe is mature with expected annual growth rates of 1- 1.5% (Berna *et al.*, 2007).

Table 1.1: Examples of HPCs and their global usage in 2011. Source: Euromonitor International, 2012.

Product category (units)		Global use of HPCs						
		Asia Pacific	Australasia	Eastern Europe	Latin America	Middle East and Africa	North America	Western Europe
Personal care products	Bar Soap ('000 kg)	1230230.4	14084.9	162308.9	582814.3	260642.7	212537	164360.8
	Body Wash/Shower Gel ('000 litres)	354930	11553	70807.4	43269.8	30947.6	164799	418683.3
	2-in-1 Products ('000 litres)	54254	737.6	27237.5	44413.4	42702.3	67123.7	85525.4
	Conditioners ('000 litres)	331920	10773.6	38839	369423.2	52961.2	181428.1	125597.7
	Standard Shampoos ('000 litres)	828378	14930	179230.3	472784.7	128161	226279.6	375595.4
	Mouthwashes/dental rinses (' 000 litres)	54128.8	8510.8	10733.8	44191.3	4782.2	160738.9	95759.8
	Toothpaste ('000 litres)	664556.5	7483.8	96390.6	207271.5	63756.3	146542.4	124844
	Mass general purpose body care ('000 litres)	61967	4239.9	28929.4	117871.2	63742.1	70848.5	117811.8
Household cleaning products	Bleach ('000 litres)	287700	15900	109200	1612100	580200	904900	1105200
	Standard powder detergents ('000 kg)	4353900	3700	932500	2759500	771000	16000	916000
	Concentrated powder detergents ('000 kg)	695300	100900	366900	8300	57500	222700	571300
	Concentrated liquid detergents ('000 kg)	400000	17900	48100	111000	10700	2043900	445600
	Bar detergents ('000 kg)	1924000	700	74500	833700	223500	800	16000
	Bathroom cleaners ('000 litres)	132800	14500	31000	36200	5900	126500	111000
	Household antiseptics/detergents ('000 litres)	143500	11100	6600	657200	38800	53600	42500

Another important group of DTD chemicals are compounds found in pharmaceutical products. There are more than 3,000 different active substances used as medicines, including painkillers, antibiotics, contraceptives, beta-blockers, lipid regulators, tranquilizers, and impotence drugs (Richardson *et al.*, 2005). More than 553,000 tons were produced in 1993 in Germany alone (Daughton and Ternes, 1999), while 35 tons of antiepileptic carbamazepin were produced in just the U.S. in 2003 (Fent *et al.*, 2006). The pattern of consumed pharmaceuticals vary between countries since some drugs are forbidden or replaced by related drugs (Fent *et al.*, 2006), and many countries dispense drugs without prescriptions (Daughton and Ternes, 1999).

1.2. Pathway for entry of DTD into the environment including geographical differences

In discussing pathways for entry of chemicals into the natural environment, geographical differences need to be considered. In developed countries, such as Western Europe and USA, wastewater is treated through the treatment systems (von Sperling and de Lemos Chernicharo, 2002; Reemtsma *et al.*, 2006). Wastewater initially enters the sewer, where it may remain for hours or a few days, during its transport to a wastewater treatment plant or environmental release site. In most situations, the wastewater is treated before release, but in some situations the wastewater is released to surface water directly or with only minimal primary treatment. Untreated raw sewage may also enter surface waters following storm surges and flood water overflow, which may prevent sewage from entering WWTP systems. Within a typical WWTP, a portion (40-60%) of the solids is removed during primary treatment. The resulting effluent is then subjected to biological treatment and the remaining solids are removed in a final clarifier. The final effluent is subsequently released to surface water. The sludge derived from solids removed during primary treatment and final clarification is most commonly digested under anaerobic conditions if the sludge disposal involves land application (**Figure 1.1**). The fraction of DTD chemicals released to the environment in the final effluent, or that is associated with sludge solids, is a function of its partitioning behaviour, its biodegradation rate and other processes (e.g. volatilization, hydrolysis, photolysis, sequestration). Also, primary and ultimate biodegradation of chemicals takes place in

wastewater treatment. While primary biodegradation is defined as a structural change (transformation) of a chemical substance by microorganisms resulting in the loss of chemical identity; the ultimate biodegradation is related to the breakdown of chemical substance by microorganisms in the presence of oxygen to carbon dioxide, or to methane under anaerobic conditions, water and mineral salts, and the production of new biomass and organic microbial biosynthesis products. The primary and ultimate biodegradation occurs during transit in the sewer, secondary treatment in an activated sludge treatment system, anaerobic digestion of sludge, and in the mixing zone of surface water for treated effluent and untreated wastewater that is directly discharged to surface water.

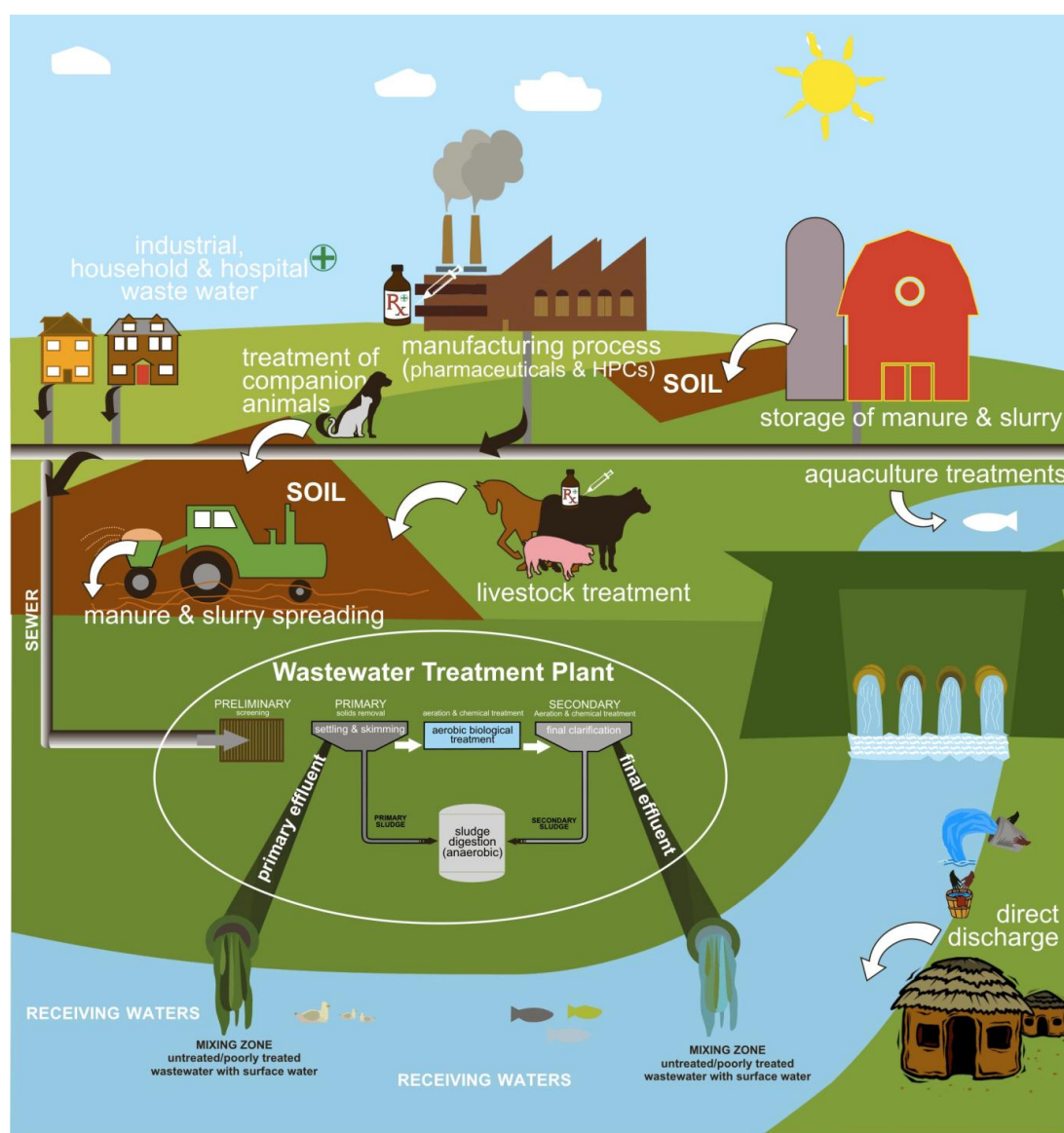


Figure 1.1: Pathways of chemical entry into the environment

Unlike pharmaceuticals, HPCs are intended for external use on the human body and are not subjected to such metabolic alterations as pharmaceuticals. Generally, HPCs enter wastewater and typically may eventually enter aquatic environments unaltered after disposal from households. During and after treatment with pharmaceuticals or veterinary medicines, humans and animals excrete a combination of intact and metabolized active chemicals, many of which are generally soluble in water and have been discharged to the aquatic environment (Richardson *et al.*, 2005). The major sources of human pharmaceuticals to the aquatic environment are domestic and hospital sewage disposal, due to widespread use of drugs for treatment of human diseases. In the case of veterinary medicines (**Figure 1.1**), chemicals can reach the surface water directly through excretion in the field. An equally important source of pharmaceuticals in soil and water, which in practice is restricted to certain countries, is the disposal of manure from intensive livestock farming sites and effluents from intensive aquaculture systems, where the use of veterinary drugs is ordinary and necessary (Boonstra *et al.*, 2011). Other sources of environmental contamination are indirect, such as use of reclaimed wastewater from treatment plants, for irrigation, sludge used in agriculture to reclaim inorganic nutrients, manufacturing waste from pharmaceutical industries, hospital wastewater and improper disposal of out-of-date medicines (Bottoni *et al.*, 2010).

Pesticides and biocides are other than DTD groups of chemicals that are of interest. They are widely used in agricultural and urban areas and they are present in soil, water and air (Barceló, 1991). The major route of pesticides and biocides into the environment is via their direct application and runoff with rainwater from treated areas to aquatic environment (Singer *et al.*, 2010).

In most regions of the world, a significant fraction of household wastewater (either untreated or treated via municipal WWTPs) will eventually reach surface waters (Whelan *et al.*, 2011). Investigations of occurrence of pharmaceuticals in aquatic environments, carried out in Austria, Brazil, Canada, Croatia, England, Germany, Greece, Italy, Spain, Switzerland, The Netherlands and the U.S., have detected more than 80 compounds, including pharmaceuticals and several drug metabolites, in the aquatic environment (Heberer, 2002). In England, Germany and Australia, the amounts of the most frequently used drugs which reach surface waters are in the hundreds of tons per year (Fent *et al.*, 2006). Pharmaceuticals can reach water

concentrations of ng/L to $\mu\text{g/L}$ and some are considered ubiquitous, such as acetylsalicylic acid which can be present in sewage effluent at concentration of 1 $\mu\text{g/L}$ (Daughton and Ternes, 1999). In the U.S., 95 organic wastewater chemicals were analysed among which veterinary and human antibiotics, prescription and non-prescription drugs were detected at concentration ranges from 0.01-10 $\mu\text{g/L}$ (Kolpin *et al.*, 2002). Antibiotic concentrations calculated and measured in hospital effluents were found to be of the same order of magnitude as the minimum inhibitory concentration of sensitive pathogenic bacteria (Hartmann *et al.*, 1999). In German hospital wastewaters ciprofloxacin concentrations ranged from 0.7-124.5 $\mu\text{g/L}$ (Hartmann *et al.*, 1999). In almost every aquatic environment in North America and Europe, pharmaceuticals, hormones, metabolites, biocides, musks, and flame retardants have been detected (Ternes, 1998; Kolpin *et al.*, 2002; Hirsch *et al.*, 1999; Hilton and Thomas, 2003). Antibiotics have also been detected in the influent as well as effluent of WWTPs and surface water in the range of ng to $\mu\text{g/L}$. For example, the anti-inflammatory drug indomethacine was found in Brazilian WWTPs at concentrations of 0.95 $\mu\text{g/L}$, 0.60 $\mu\text{g/L}$ and 0.20 $\mu\text{g/L}$ in the influent, effluent and surface water, respectively (Daughton and Ternes, 1999). Following application of settled sewage, chemicals can be introduced into soil and they can be present in concentrations of up to several hundred $\mu\text{g/kg}$. They are also detected in sediments after use in fish farming (Kümmerer *et al.*, 2004).

In regions of the world with poor provision of wastewater treatment, such as Asia, South America and Africa raw sewage is often discharged untreated into surface waters (Finnegan *et al.*, 2009, Whelan *et al.*, 2007). For example, in 2002, more than 90% of the population of Brazil was without connection to municipal wastewater treatment plants. As a consequence, surface waters continuously receive considerable amounts of untreated domestic sewage containing surfactants as a major constituent. Such polluted waters are of specific concern if they are used as a source of drinking water (Eichhorn *et al.*, 2002) but can also have significant ecological effects.

1.3. Ecotoxicological implications of chemical release into the environment

Ecotoxicological data on chemicals are vital to determine a predicted non-effect concentration (PNEC). At the same time, the environmental behaviour of the substance can be determined from measured or test data to derive a predicted environmental concentration (PEC). PEC and PNEC are compared and if the PEC is higher than the PNEC, it is concluded that the test substance may constitute an environmental risk (Pagga, 1997). Experimental evidence indicates that pharmaceuticals and HPCs may cause harmful effects, such as growth, development, morphological, metabolic and sex alteration on invertebrates, fish, amphibians, algae and plants, as well as induction of antibiotic resistance in aquatic pathogenic microorganisms, and disruption of biodegradation activities in WWTPs (Bottoni *et al.*, 2010; Brausch and Rand, 2011). Chemicals which are mutagenic, carcinogenic e.g. formaldehyde, 4-aminobiphenyl, or bioaccumulative e.g. polychlorinated biphenyls (PCBs), may also have implications for humans (Swenberg *et al.*, 1983; Shelby, 1988; Schell *et al.*, 2008).

In a Norman bulletin Issue No.3 a watch list of emerging chemicals for surface water monitoring, was defined for Europe (Botta *et al.*, 2012). About 2,400 compounds were considered as potential candidate substances, of which more than 400 are pharmaceuticals and associated metabolites, and about 70 are chemicals used in personal care and household products. 700 chemicals of the starting list of compounds are already part of monitoring programmes performed by water agencies. The remaining chemicals were mainly pesticides, biocides and industrial chemicals. So far, beta-sitosterol, 17- β -estradiol, closantel, dimethicone, penfluridol, prochloroperazine and bithionol received the highest scores on the priority list. Sui *et al.* (2012) reported 17 pharmaceuticals which were of concern as priority pollutants. Among them erythromycin, diclofenac acid and ibuprofen were regarded as high priority pharmaceuticals present and treated in WWTPs.

The environmental fate and effects of pharmaceuticals and most HPC chemicals are poorly documented, although considerable persistence and bioaccumulation in aquatic organisms has been reported for some. For instance, Daughton and Ternes

(1999) reported iopromide as extremely persistent, and bioconcentration of methylbenzylidene camphor and sunscreens used in UV protection products, was demonstrated in roach from German lakes. A range of endocrine disrupting chemicals (EDCs) can be present in WWTP effluent and may have impact on natural ecosystems as well as human health (Ying *et al.*, 2003). The steroid estrogen, 17 α -ethinylestradiol (an active ingredient in birth control pills), belongs to an important group of trace organic chemicals detected in WWTP effluents in developed countries (Xu *et al.*, 2012; Shappell *et al.*, 2008) which may lead to sex change in some fish affecting their reproduction and survival (Nash *et al.*, 2004). According to Breitholtz *et al.* (2003) nitro musks can affect the growth rate and reproduction of herpacticoid copepod *Nitocra spinipes*.

Widespread use of antibiotics is a major cause of spreading resistance among bacterial pathogens and sufficiently high concentrations could also have acute effects on bacteria. Exposure to antibiotics may alter the structure of microbial community and thereby affect the higher food chain (Daughton and Ternes, 1999). The commonly used pharmaceuticals ibuprofen, naproxen, ketoprofen, diclofenac and clofibric acid were found to influence the structure of activated sludge bacterial communities in small-scale pilot wastewater treatment reactors, where a reduced diversity of bacterial communities was observed in the reactors supplied with 50 $\mu\text{g/L}$ of each pharmaceutical (Kraigher *et al.*, 2008).

Trace substances could also lead to serious problems if they are susceptible to biomagnification (the increase in concentration of a chemical that occurs in a food chain) and are subsequently toxic to species at higher trophic levels in food chains. For instance, Evans *et al.* (1991) reported biomagnification of PCBs, toxaphene, and dichlorodiphenyltrichloroethane (DDT) in Lake Michigan offshore food web. Wide use of DDT impacted the reproduction and survival of Bald Eagles (Grier, 1982). In addition, organic chemicals can impart objectionable tastes and odours to water at levels of ng/mL (Boethling and Alexander, 1979). Hence, legislation has been developed to control and prohibit the manufacture, importation, distribution, and sale of domestic and personal care products containing chemicals that are persistent, bioaccumulative and toxic (PBT), and to support the development of more environmentally friendly chemicals. Efforts were made to prioritise existing

chemicals based on the following criteria; (1) inherent properties (the priority should be given to PBT or very persistent, very bioaccumulative chemicals (vPvB)), (2) volume (tonnes/year supplied in the EU to uses in the scope of authorisation) and (3) wide-dispersive use which refers to many small point sources or diffuse release (ECHA, 2010).

1.4. Regulatory drivers for chemical management

The growth and development of the Chemical Industry has generated large numbers of new chemical substances. Their application (e.g. in production of household and personal care products) has resulted in their eventual release into environmental compartments. Therefore, regulation is required for monitoring of existing and new substances, their application, amounts used and concentrations measured in the environment.

1.4.1. REACH and PBT assessment

The former European Commission legislative framework for chemical substances consisted of many different Directives and Regulations which has developed historically. There were different rules for existing chemicals (chemicals reported as being on the market between 1 January 1981 and 18 September 1981) and new chemicals (chemicals introduced to the market after 1981). This system also did not produce sufficient information about the effects of the majority of existing chemicals on human health and the environment. Moreover, the former system restricted research and innovation, causing the EU chemicals industry to be delayed in comparison with the US and Japan in this regard (European Commission, 2007). The new European chemicals legislation, Registration, Evaluation and Authorisation of Chemicals (REACH) was adopted in December 2006 and it came into force in 1 June 2007 (ECHA, 2008). This legislation aims to improve risk management of industrial chemicals produced in or imported into Europe to protect human health and the environment from potentially hazardous classes of chemicals. This is achieved by generating data on the toxicity and ecotoxicity of industrial chemicals (HSE, 2012) and by providing a defined set of information, in the form of a

registration dossier, to the Chemicals Agency by manufacturers and importers of substances (ECHA, 2008). At the same time REACH aims at stimulating innovation towards the design of safer chemicals and processes, thus enhancing corporate responsibility as well as promoting competition within the European Chemical Industry (Rudén and Hansson, 2010; Lewis *et al.*, 2007, ECHA, 2008). The type of data that need to be generated in order to serve the main policy objectives of the REACH framework are classified into the following categories: (1) key physical and thermochemical properties of substances, (2) biological activity of chemical substances, (3) data associated with the ecological effects and environmental fate of chemical substances. These data are generated through laboratory tests and experimental studies as well as the establishment of quantitative structure relationships (SARs) or quantitative structure-activity relationships (QSARs) (Lewis *et al.*, 2007).

Information on the biodegradability of chemicals is an essential requirement for hazard assessment (e.g. for classification and labelling), environmental risk assessment (for chemical safety assessment) and persistency assessment (ECETOC, 2007). Environmental hazard assessment (hazard identification and hazard characterisation) involves gathering or generating and evaluating data of chemical substances and concluding on their inherent eco-toxicological effects and environmental fate. Risk assessment aims to estimate or predict the extent of exposure of chemicals to the target species and/or the environment through its production, use and disposal, while persistency assessment is focused on whether a chemical has got potential to persist in the environment (OECD, 2013). International and national frameworks have been developed to identify PBT substances and to control their use and emission to the environment. In Europe, the set of guidance was developed under REACH according to which chemicals that are registered must include chemical safety assessment (CSA) for all substances manufactured within or imported into the European Union (EU) in quantities exceeding 10 tonnes/year. The CSA includes (1) human health hazard assessment, (2) assessment of physico-chemical properties, (3) environmental hazard assessment, and (4) PBT assessment including identification of vPvB substances (Boethling *et al.*, 2009). In China, guidelines were created in order to implement the Environmental Protection Law of the People's Republic of China, to protect the environment, to protect human health

and standardize the work of risk assessment for chemical substances. The guidelines specify the principles, content, procedures, methods and technical requirements of chemical substance risk assessment. They were drawn up by the Division of Science and Technology under the Ministry of Environmental Protection (MEP) and were approved by the MEP. Under the environmental management system in China, chemicals are divided into existing chemical substances and new chemical substances by the IECSC (Inventory of Existing Chemical Substances of China) (MEP, 2011). Under the guidance of the United Nations Environment Programme (UNEP) there was global agreement to prohibit, restrict, or reduce the production, use, or release of certain persistent organic pollutants (POPs) which resulted in the Stockholm Convention being adopted in 2001. Similar regional negotiations were completed under the United Nations Economic Commission for Europe (UNECE), Convention of Long-Range Transboundary Air Pollution (LRTAP), the North American Commission for Environmental Cooperation, Sound Management of Chemicals (NACEC-SMOC) Initiative and the United States/Canada agreement to control the discharge or release of POPs in the Great Lakes Basin of North America (Boethling *et al.*, 2009). The United States Environmental Protection Agency (EPA) also developed a multimedia strategy for PBT pollutants (US EPA, 2012). However, in EU REACH Regulation places greater responsibility on industry to manage the risks from chemicals and to provide safety information on the substances. Manufacturers and importers are required to gather information on the properties of their chemical substances, which will allow their safe handling, and to register the information in a central database run by the European Chemicals Agency (ECHA) in Helsinki (European Commission, 2007).

Persistence of chemicals is one of the main issues in the environmental risk assessment and it is associated with chemical resistance to environmental degradation through chemical, biological, and photolytic processes. Persistent chemicals are defined as chemical substances that persist in the environment, bioaccumulate through the food web, and pose a risk of causing adverse effects to human health and the environment (Stockholm Convention, 2008). These concerns lead to Stockholm Convention which was signed in 2001. According to Thouand *et al.* (2011), persistence is not only regarded as a property of a substance, but also as the capability of various environmental samples to degrade a substance under

realistic exposure conditions and the ratio between food and biomass (F/M ratio). Understanding the mechanisms responsible for chemical persistence (intrinsic, environmental and physical persistence) is also important for the persistency assessment. Under REACH a tiered approach for the persistency evaluation was developed (Thouand *et al.*, 2011) and it usually involves: (1) the rates of removal of a substance by physical, biological and chemical processes occurring in soil, water and sediment (2) standardized test methods for testing persistence/biodegradation, and (3) QSARs which are used to estimate half-lives when no experimental data exist (Boethling *et al.*, 2009). In the present European Technical Guidance Document (TGD) the following persistency criteria are mentioned; test for ready biodegradability, test for inherent biodegradability, and estimated half-life data (European Commission, 2003).

Although, few persistence criteria exist (e.g. 60 days as the half-life threshold for persistence in water) and are used in the frameworks mentioned above (Widley *et al.*, 2004), the evaluation of persistence is challenging under environmental conditions. Scheringer *et al.* (2006) identified five problems associated with the persistence evaluation procedure as it will be implemented under the REACH legislation; (1) insufficient data are required under REACH to apply the persistence criteria, (2) the half-life criteria are not comprehensive because they do not include a criterion for persistence in air, (3) the selection of the the half-life criteria is not optimal because they are based on the half-lives of certain reference chemicals, (4) half-lives estimated from chemical structure are probably not sufficient for classifying a chemical as persistent, and (5) long-range transport, which is a problem occurring on a shorter time scale than local contamination by persistent chemicals, is not addressed at all. Hence, the possibility exists that some chemicals which are persistent in the environment will not be identified as persistent under REACH.

1.4.2. European Union and REACH

In the European Union (EU) the legislation addressing industrial chemicals began with the Dangerous Substances Directive 67/548/EEC (DSD) enacted in 1967, which specified the management requirements for the classification, packaging and

identification of hazardous chemicals. In 1993 Regulation 793/93/EEC (the Existing Substances Regulation (ESR)) introduced a comprehensive framework for evaluation and control of existing substances. In 1995, to support the environmental management of chemicals, the EU issued the first edition of Technical Guidance Document (TGD) in Risk Assessment. Assessment of existing substances was carried out mainly by the member countries until the promulgation of the REACH regulation (Wang *et al.*, 2012).

1.5. Methods for determining biodegradation rates

1.5.1. Predictive modelling

Prediction of chemical biodegradability in the natural environment is of high interest among regulators, chemical producers and users and a range of predictive methods have been developed. One example is CATABOL, a computer software for biodegradability prediction (Jaworska *et al.*, 2002). This program is based on an expert system which calculates probabilities of the individual chemical transformation. CATABOL software has been used to predict the extent of chemical biodegradation, while also generating information about the metabolic pathway and identification of stable metabolites, during the Organization for Economic Cooperation and Development (OECD) test 302 (Japanese Ministry of International Trade and Industry (MITI (I) test) using a database created for 532 chemicals (Jaworska *et al.*, 2002). Another group of models are the BIOWIN models, which include the linear and non-linear BIODEG and MITI models for estimating the probability of rapid aerobic biodegradation and an expert survey model for primary and ultimate biodegradation estimation (Posthumus *et al.*, 2005). In MITI model the probability of rapid biodegradation is estimated by means of multiple linear or nonlinear regression against counts of 36 chemical substructures (molecular fragments) plus molecular weight. A total dataset of 884 chemicals was compiled to derive the fragment probability values. The dataset consists of 385 chemical that were critically evaluated as readily degradable and 499 chemicals that were critically evaluated as not readily biodegradable (Tunkel *et al.*, 2000). Boethling *et al.* (2003) reported validation of the six BIOWIN models for predicting ready biodegradability

of premanufacture notice chemicals (PMNs) based on a database containing 305 structures after review of more than 10,000 individual PMNs. BIOWIN models were found useful for screening-level applications to predict not-readily biodegradable substances with high accuracy and distinguish them from readily biodegradable chemicals (Boethling *et al.*, 2003; Posthumus *et al.*, 2005). It is noteworthy that there predictions of chemical toxicity and estimation of chemical persistence using environmental models are based on the chemical structure and physico-chemical properties, and they do not consider complex environmental conditions which may affect chemical toxicity and fate (Cronin and Livingstone, 2004). Extrapolation of such data into the environment may lead to inaccurate risk assessment.

1.5.2. OECD tests

1.5.2.1. Role of biodegradation testing

In order to assess the environmental risk of particular chemicals, information allowing the estimation of their likely concentrations in the environment is necessary for hazard and risk assessment (OECD Guideline for testing of chemicals, 2005). The biodegradability of chemicals is one of the most important aspects determining environmental behaviour and is required for chemical classification purposes (Rudén and Hansson, 2010). Chemicals which are not biodegradable remain in the environment as persistent substances, and may pose a risk for bioaccumulation in living organisms and other environmental compartments. Such an estimate of biodegradability must initially be based on knowledge of the likely use and disposal patterns of the chemical (**Figure 1.1**), its physico-chemical properties and the characteristics of the receiving environment. OECD test results are also used as input into exposure assessment, developing sampling and analysis protocols for related field studies and model development.

1.5.2.2. Historical aspects and principal design of OECD tests

A range of methods for investigating and monitoring biodegradation processes have been developed to predict the fate of chemicals in the environment. Most efforts have focused on the fate of chemicals in the aquatic environment, especially in

wastewater treatment processes (Reuschenbach *et al.*, 2003). Testing biodegradability under laboratory conditions aims to obtain a reliable prediction of the biodegradability of substances in the environment (Pagga, 1997). Over 30 years ago, in 1981, OECD first published its guidelines for testing the biodegradation of chemicals, which were updated in 1993 (OECD Guidelines for testing of chemicals, 1993). In 1990, a classification in accordance with the OECD was proposed (OECD, Paris). Three groups of tests were defined: (1) ready biodegradability (or screening), (2) inherent biodegradability and (3) simulation (Lapertot and Pulgarin, 2006). However, the OECD tests were not designed to measure chemical persistence or environmental biodegradation half-lives, and were validated using only readily biodegradable chemicals (Comber and Holt, 2010). A list of reference chemicals for use as positive and negative controls in standardized biodegradability tests (**Table 1.2**) has been proposed by regulators and industry within an agreed set of properties and characterized set of biodegradability behaviour, which cover a range of environmental persistence and non-persistence (Comber and Holt, 2010). These chemicals group into bins (**Table 1.2**), which align with OECD tiered testing (**Figure 1.2**) and represent the relationships between the screening and higher tier tests together with potential regulatory impact from REACH (Comber and Holt, 2010).

Table 1.2: Reference chemicals and their classification into BINs. Source: Comber and Holt, 2010.

BIN	Description	Half-life (days)	Chemical example
1	Reference chemicals which would normally pass a RBT or modified RBT test	< 15	aniline, sodium benzoate, phenol
2	Reference chemicals that would normally pass an enhanced screening biodegradability test but currently fail any other tests	16-40	4-chloroniline, 4-fluorophenol, 1,2,3-trimethylbenzene
3	Reference chemicals that would normally fail any biodegradability screening test whether modified RBT or enhanced screening biodegradability test	41-60	o-terphenyl, cyclodecane, dibutylphenol
4	Reference chemicals that should never pass a modified RBT or an enhanced biodegradability screening test	> 60	hexachlorobenzene, benzo(a)pyrene, hexachlorohexane

Where: RBT- ready biodegradability tests

The modified ready biodegradability tests and enhanced biodegradability screening tests are two types of screening tests which were identified beyond the OECD 301 series (OECD Guidelines, 1993). The modified screening tests were designed to test (1) the biodegradability at low test substance concentrations, when the test substance is known or expected to exert toxicity to the microbial inoculum, and (2) to test biodegradability of poorly water-soluble substances (Comber and Holt, 2010). The enhanced biodegradability tests include a number of potential enhancements to the ready biodegradability test; (1) increased test duration, (2) testing in larger vessels, (3) increasing the biomass concentration, (4) low level pre-adaptation test systems, and (5) semi-continuous biodegradability tests. The enhancements were designed to help improve the environmental relevance of biodegradability assessments without the immediate requirement for simulation level testing (Comber and Holt, 2010).

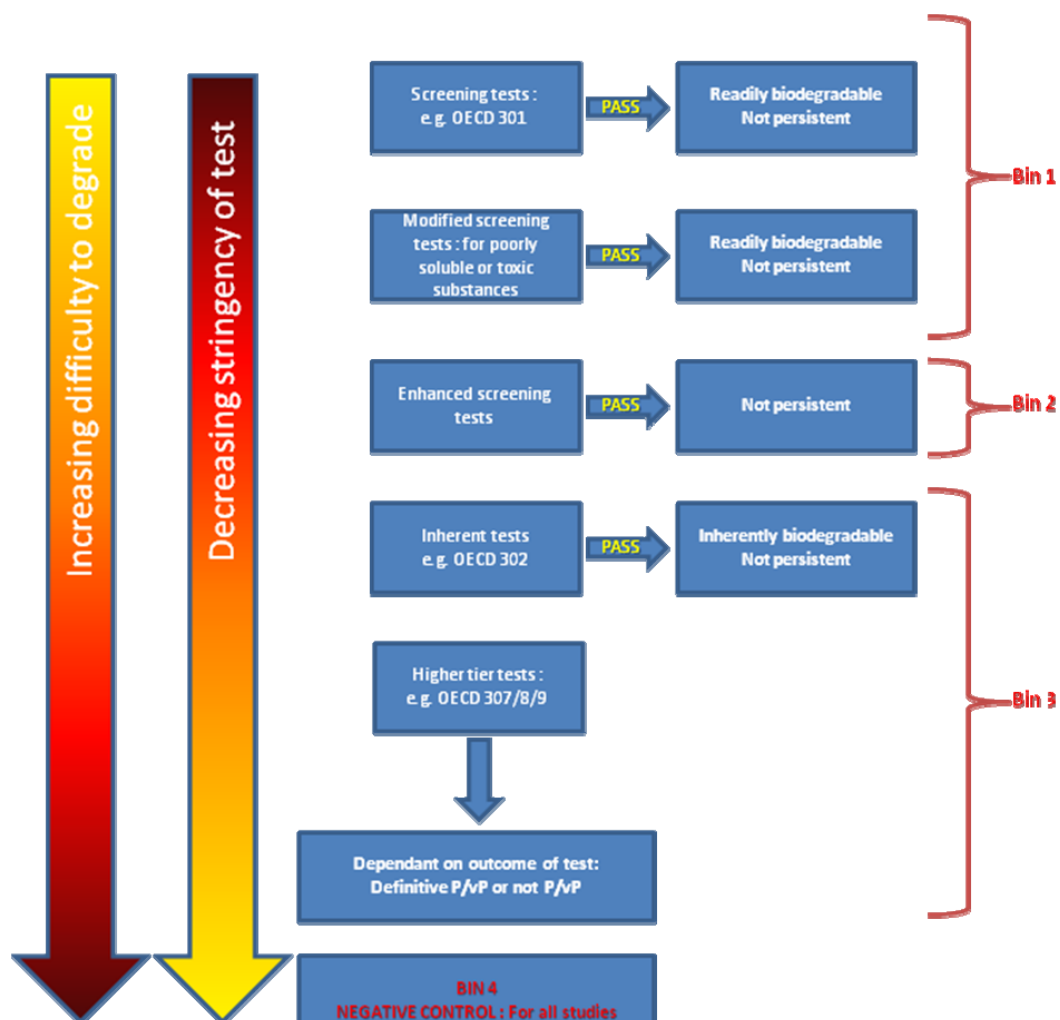


Figure 1.2: Relationship between screening and higher tier biodegradability tests and the bins.

Source: Comber and Holt, 2010.

1.5.2.3. Overview of current tests

The choice of a test normally depends on the testing purpose, and under REACH, the testing is graded according to production level and properties of a substance (Pagga, 1997).

1.5.2.3.1. Ready biodegradability tests (screening tests)

Ready biodegradability tests are considered a stringent first tier which offers limited opportunities for biodegradation and acclimation of the inoculum (Reuschenbach *et al.*, 2003). Screening tests are conducted under aerobic conditions, in which a high concentration of the test substance (in range of 2 to 10 mg/L) is used (OECD Guidelines for testing of chemicals, Test No. 301, 1993). Six methods permit the screening of chemicals for ready biodegradability in an aerobic aqueous medium and they are based on the removal of organic compounds measured as dissolved organic carbon (DOC) (OECD 301 A, 301 E), the production of the catabolic end-product carbon dioxide (OECD 301 B) and the determination of the biochemical oxygen demand (BOD) (OECD 301 C, 301 D, 301 F) (Reuschenbach *et al.*, 2003). It is required that the pass level of ready biodegradability is reached within 10 days of the start of biodegradation (10-day window) within the 28-day period of the test. The pass levels depend on the analytical parameter measured. These are: 70% DOC removal (OECD 301 A and 301 E); 60% theoretical carbon dioxide (ThCO₂) (OECD 301 B), and 60% theoretical oxygen demand (ThOD) (OECD 301 C, 301 D and 301 F) (OECD Guidelines for testing of chemicals, Test No. 301, 1993). According to OECD Guidelines (OECD 301) RBT tests also differ in terms of the inoculum size. In OECD 301 A, 301 B and 301 F inoculum size is 10⁷-10⁸ cells/L, in OECD 301 E concentration of inoculum is approximately 10⁵ cells/L, while OECD 301 D and 301 C have inoculum size of 10⁴-10⁵ and 10⁷-10⁸ cells/L, respectively. Screening tests indicate if a compound is degradable under natural conditions and a pass indicates that the chemical is degradable although, they often underestimate the potential of degradation in environmental systems (Lapertot and Pulgarin, 2006; Comber and Holt, 2010; OECD Guidelines, 1993). A positive result in a test for ready biodegradability can be considered as indicative of rapid and ultimate degradation in most aqueous environments including biological WWTPs. A negative result does not

necessarily mean that the chemical will not be degraded under relevant environmental conditions, but the next level of testing (i.e. either a simulation test or an inherent biodegradability test) should be considered (OECD Guidelines, 2005).

1.5.2.3.2. Inherent (potential) biodegradability tests

The second tier tests are inherent tests, which are inoculated with a high concentration of both the target compound and inoculum, usually activated sludge (with inoculum size of 10^7 - 10^8 cells/L), under aerobic conditions (Comber and Holt, 2010). Inherent biodegradability can be measured by specific analysis (primary biodegradation) or by non-specific analysis (ultimate biodegradation). Specific methods used for inherent biodegradability tests include: Modified SCAS Test (OECD 302 A), Zahn-Wellens/EMPA Test (OECD 302 B) and Modified MITI Test (II) (OECD 302 C). Limits for inherent test duration and biodegradation rates are not defined. Biodegradation above 20% of theoretical (measured BOD, DOC removal or chemical oxygen demand (COD)) may be regarded as evidence of inherent, primary biodegradability, whereas biodegradation above 70% of theoretical (measured as BOD, DOC removal or COD) may be regarded as evidence of inherent, ultimate biodegradability. The test procedure offers a higher chance of detecting biodegradation compared to tests for ready biodegradability, and therefore, if an inherent test is negative this could indicate a potential for environmental persistence (Comber and Holt, 2010).

1.5.2.3.3. Simulation tests

Simulation tests aim at assessing the rate and extent of biodegradation in a laboratory system designed to represent either the aerobic treatment stage of a WWTP or environmental compartments, such as fresh or marine surface water. These are aerobic and anaerobic tests that provide data for biodegradation under specified environmentally relevant conditions. They use indigenous biomass, media, and relevant solids (i.e. soil, sediment, activated sludge or other surfaces) to allow sorption of the chemical and a typical temperature that represents the particular environment. Therefore, inoculum size may vary from 10^4 - 10^5 to 10^7 - 10^8 cells/L depending on the test. A representative and low concentration of test substance is

used in these tests (e.g. less than 1 µg/L to 100 µg/L), which is low enough to ensure that the biodegradation kinetics reflect those expected in the environment being simulated. The fate of chemicals in WWTPs can be studied in the laboratory by using the Simulation Test – Aerobic Sewage Treatment: Activated Sludge Units (OECD 303 A) and Biofilms (OECD 303 B). The removal of the test substance is determined by monitoring the changes in DOC and/or COD. The following tests are used to simulate the biodegradation of organic chemicals under environmentally realistic conditions in soil, sediment or surface water: Aerobic and Anaerobic Transformation in Soil (OECD 307); Aerobic and Anaerobic Transformation in Aquatic Sediment Systems (OECD 308); Aerobic Mineralisation in Surface Water – Simulation Biodegradation Test (OECD 309); and simulation tests to assess the biodegradation of chemicals discharged in wastewater (OECD 314) (Comber and Holt, 2010). A diagram describing OECD 314 is presented in **Appendix Figure 0.1**.

1.5.2.3.3.1. Aerobic and anaerobic transformation in soil (OECD 307)

OECD 307 was designed for evaluating transformation of chemicals in soil. The experiments are performed to determine the rate of transformation of the test substance and the nature and rates of formation and decline of transformation products to which plants and soil organisms may be exposed. Such studies are required for chemicals which are directly applied to soil or which are likely to reach the soil environment. The results of such laboratory studies can also be used to develop sampling and analysis protocols for related field studies. Test duration should not exceed 120 days due to decrease of soil microbial activity with time. Estimation of test substance transformation half-life, DT_{50} is performed (OECD Guidelines for testing of chemicals, Test No. 307, 2002).

1.5.2.3.3.2. Aerobic and anaerobic transformation in aquatic sediment systems (OECD 308)

OECD 308 is used to assess transformation of chemicals which can enter shallow or deep surface waters by such routes as direct application, spray drift, run-off, drainage, waste disposal, industrial, domestic or agricultural effluent and

atmospheric deposition. As a result half-life, DT_{50} and if appropriate, DT_{75} and DT_{90} of the test substance are calculated and pseudo first-order kinetics are applied to obtain information on the rate of test substance dissipation in the water and sediment (OECD Guidelines for testing of chemicals, Test No. 308, 2002).

1.5.2.3.3.3. Aerobic mineralisation in surface water (OECD 309)

The purpose of OECD 309 is to measure the time course of biodegradation of a test substance at low concentration in aerobic natural water (fresh, brackish or marine) and to quantify the observations in the form of kinetic rate expressions. This simulation test is a laboratory shake flask batch test based on the ISO/DIS 14592-1 and it also includes elements from OECD Guidelines 307 and 308. With long test times, semi-continuous operation replaces batch operation in order to prevent deterioration of the test microcosm. The principal objective of the test is to determine the mineralisation of the test substance in surface water, and mineralisation constitutes the basis for expressing degradation kinetics. An optional, secondary objective of the test is to obtain information on the primary degradation and the formation of major transformation products. Test substance is applied at low concentration (e.g. less than 1 µg/L to 100 µg/L) which is low enough to ensure that the biodegradation kinetics obtained in the test reflect those expected in the environment. First order kinetics are normally expected in this test. However, there may be circumstances where other kinetics are more appropriate (OECD Guidelines for testing of chemicals, Test No. 309, 2004).

1.5.2.3.3.4. Simulation tests to assess the biodegradation of chemicals discharged in wastewater (OECD 314)

OECD 314 guideline describes methods for determining the extent and kinetics of primary and ultimate biodegradation of organic chemicals whose route of entry into the environment begins with their discharge to wastewater. The five tests were designed to assess biodegradation during key phases of wastewater transit as well as treatment and environmental release. They include elements from OECD Test

Guidelines 301, 303A, 309, 310 and 311. The principal objectives of the methods are to: (1) measure the rate of primary biodegradation, (2) measure the rate of mineralization, and (3) follow the formation and decay of major transformation products. These tests are intended as higher tier tests for assessing the biodegradation of chemicals which do not biodegrade in OECD screening tests, or for refining biodegradation rates used for an exposure assessment (OECD Guidelines for testing of chemicals, Test No. 314, 2008). Reproducibility and reliability of all the tests were improved consequently with the use of standardized protocols, which list experimental conditions, analytical methods and criteria for whether a chemical is considered to be biodegradable (pass) or non-biodegradable (fail) (Howard *et al.*, 1987).

1.6. Environmental realism of tests

There are OECD tests simulating aquatic (OECD 309, OECD 314) and terrestrial compartments (OECD 307), based on static, semi-continuous, or continuous principles, operated under aerobic or anaerobic conditions with different test media. The test conditions should be standardized as far as possible and they should reflect the environmental realism of biodegradation processes. Although, simulation tests aim to reflect environmental conditions and are applicable for use in higher tier biodegradation test, their complex test design, long test duration and high costs, make them not suitable as routine tests (Merrettig-Bruns and Jelen, 2009). Often, screening and to some extent simulation OECD tests are conducted without consideration of (1) environmental context, (2) water chemistry, (3) inoculum concentration (quantity) and composition (diversity/quality), (4) test substance concentration, and (5) global relevance (e.g. biogeography).

1.6.1. Effect of environmental factors on biodegradability assessment

Screening tests discriminate readily biodegradable chemicals from persistent chemicals. These chemicals are further tested in OECD simulation tests which often underestimate the potential of chemical degradation in environmental systems (Guhl and Steber, 2006). This could be due to a variety of factors, which differ between lab

and natural environments for instance, pH, light/dark cycle, oxygen, flow rate of water body and river bed form. **Table 1.3** and **1.4** present factors occurring in natural environments and how they could potentially impact the biodegradation of chemicals in laboratory conditions. For example, light conditions are excluded from biodegradation tests to avoid the growth of algae, which may affect the biodegradation of tested chemicals and/or make the interpretation of CO₂ data analysed in such tests difficult due to fixation of the CO₂. Interestingly, some authors have reported that light enhances biodegradation of chemicals (Borde *et al.*, 2003; Li *et al.*, 2005) due to growth of phototrophic organisms capable of chemical biodegradation (Roldán *et al.*, 1998; Lima *et al.*, 2003; Thomas and Hand, 2011). Temperature is also an important environmental variable which affects bacterial growth and biomass (White *et al.*, 1991). Temperature has a strong influence on the period of acclimation of microorganisms and on the rate of biodegradation (Kang and Kondo, 2002; Manzano *et al.*, 1999) therefore, it may affect the outcome of biodegradation tests. pH may vary widely within and between environmental compartments and may alter the toxicity of environmental pollutants (Rutgers *et al.*, 1998) and affect the biodegradation rate (Li *et al.*, 2008). Furthermore, chemicals can be differentially mineralized in aerobic and/or anaerobic environmental compartments (Boyd *et al.*, 1983; Battersby and Wilson, 1989). In natural river systems attenuation may be influenced by sediment dynamics and flow velocity which determine contact of chemicals with bacteria on the sediment surface (Kunkel and Radke, 2008). The size and structure of biofilm is also affected by flow shear stress (Lau and Liu, 1993; Battin *et al.*, 2003). Limited river biofilm formation may disturb self-purification processes occurring in rivers since river biofilms play crucial role in the removal of contaminants from the water (Muia *et al.*, 2003; Edwards *et al.*, 1990).

Table 1.3: Environmental variables, temperature and light, affecting the biodegradation of chemicals.

Variables	Test compound and its concentration	Degradation test + results	Reference
pH	<i>Para</i> -nitrophenol (600mg/L)	Mineralization of <i>para</i> -nitrophenol by isolate <i>Arthrobacter</i> sp. Y1 within 120 hours (degradation rate affected by pH, optimum pH=8-9)	Li <i>et al.</i> , 2008
	Pentachlorophenol (PCP)	Toxicity and degradation of PCP is affected by pH	Rutgers <i>et al.</i> , 1998
	Hexachlorocyclohexane	Temperature and pH effect on chemical degradation (optimum conditions were found for biodegradation at pH =8 and T=30°C)	Siddique <i>et al.</i> , 2002
Temperature	Bisphenol A	Longer degradation at 20°C than at 30°C was observed with half –lives from 4-7 days to 2-6 days, respectively	Kang and Kondo, 2002
	Nonylphenol polyethoxylate	Temperature has a strong influence on the period of acclimation of the microorganisms and on the rate of biodegradation (biodegradation varied from 68-96% at 7 and 25°C, respectively)	Manzano <i>et al.</i> , 1999
Light	<i>Para</i> -nitrophenol (400 mM)	Light degradation of <i>para</i> -nitrophenol by <i>Rhodobacter capsulatus</i>	Roldán <i>et al.</i> , 1998
	<i>Para</i> -nitrophenol (50 mg/L)	Biodegradation of <i>para</i> -nitrophenol by microalgae (total degradation within 5 days)	Lima <i>et al.</i> , 2003
	<i>Para</i> -nitrophenol (5 mg/L, at pH=4.0)	Photocatalytic degradation of <i>para</i> -nitrophenol on nanometer size titanium plates (44-80% degraded)	Li <i>et al.</i> , 2005
	Salicylate, phenol, phenanthrene	Photosynthesis-enhanced biodegradation of aromatic pollutants by algal-bacterial microcosms	Borde <i>et al.</i> , 2003
	2,4-dinitrophenol (2,4-D)	Biodegradation of 2,4-D with mixed culture of phototrophic microorganisms	Hirooka <i>et al.</i> , 2006
	Continuous light	(total degradation in the dark, other conditions caused accumulation of metabolite 2-amino-4-nitrophenol (2-ANP)	
	Light/dark cycle (8 hours/16 hours)		
	Continuous darkness		

Table 1.4: Environmental variables, oxygen and bed form, affecting the biodegradation of chemicals.

Variables	Test compound and its concentration	Degradation test + results	Reference
Oxygen	Phenol	Anaerobic degradation in digested sludge (majority monosubstituted phenols degraded)	Boyd <i>et al.</i> , 1983
	Phenol, benzoates, phthalic acid esters, pesticides, homocyclic and heterocyclic ring compounds, monosubstituted benzenes	Anaerobic degradation of different chemical groups of compounds. Only pyridine and quinoline degraded, 1-and 2-naphthol and anthraquinone had inhibitory effect on digesting sludge	Battersby and Wilson, 1989
	<i>Para</i> -nitrophenol (500 mg/L)	Aerobic degradation by activated sludge (acclimated biomass degraded PNP within 11 hours)	Bhatti <i>et al.</i> , 2002
Bed form	Diclofenac, bezafibrate, ibuprofen, naproxen, gemfibrozil	The effect of flow velocity and sediment dynamics (water-sediment interactions; flat sediment vs. moving sediment) on the attenuation of acidic pharmaceuticals. (Majority degraded within 2.5- 18.6 days while gemfibrozil degraded after 10.5 days)	Kunkel and Radke, 2008

1.6.2. Water chemistry

The impact of untreated wastewater on aquatic ecosystems has been studied for many years. The severity of this impact depends on the dilution of the receiving water. The zone of impact can be characterized by low dissolved oxygen (DO) and high ammonia (NH_3) concentrations, which can be inhibitory to aquatic organisms (McAvoy *et al.*, 2003). Aquatic organisms found along an impacted river may have adapted to break down chemicals which they may use for growth and energy. This is known as self-purification. Prediction of the extent of the self-purification processes could be used to assess the potential effects of a consumer product ingredient that may cause an effect over and above the impact caused by conventional chemicals originating from human waste (McAvoy *et al.*, 2003). Surface waters, such as rivers, often serve as disposal systems for wastes coming from residential areas, industries, and manufacturing plants. Elevated levels of organic wastes from these sources cause consumption of DO and production of ammonia due to biological decomposition (**Figure 1.3**). “Recovery” of biotic integrity is dependent on the concomitant increase of DO and decrease of NH_3 . A risk assessment of the fate of test substances in rivers that receive untreated wastewater should take into account the potential adverse effect of increased BOD and NH_3 on biodegradation processes (Dyer *et al.*, 2003). In many regions around the world, industrial and domestic wastewaters are disposed directly into the environment with no prior treatment. Under this direct discharge condition receiving water will assimilate the wasteload to a certain capacity. If the assimilative capacity is exceeded, an impact will occur. This impact zone will extend down the river until the waste load is assimilated by physical, chemical and biological processes (Peng *et al.*, 2000). Whelan *et al.* (2007) studied the behaviour of linear alkylbenzene sulphonate (LAS) under direct discharge conditions in a surface water system in Vientiane (Lao PDR). They reported changes in the ratios of LAS alkyl chain homologues with a preferential removal of longer chain lengths. The concept of the impact zone is used in simulation biodegradation tests (OECD 314e, Finnegan *et al.*, 2009), which aim to estimate the fate of chemicals under the direct discharge conditions. This approach results in more environmentally realistic data which enables extrapolation to natural environments where such scenario occurs and may be useful for better ecological risk assessment (McAvoy *et al.*, 2003) and understanding the behaviour of chemicals

(Whelan *et al.*, 2007) under direct discharge conditions.

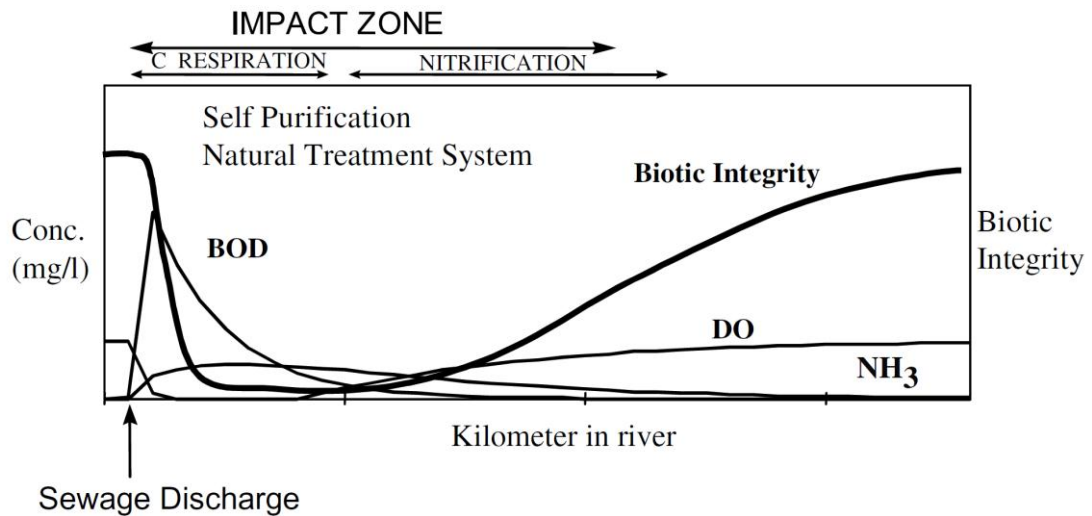


Figure 1.3: Relationship of an impact zone caused by the direct discharge of organic wastes, as described by increased BOD, NH_3 and decreased DO, with biotic integrity. Biotic integrity refers to the condition of aquatic algal, invertebrate and fish communities. Source: Dyer *et al.*, 2003.

1.6.3. Inoculum

According to Thouand *et al.* (1996) at least three parameters must be controlled when running a biodegradation test. These are the physico-chemical conditions (pH, temperature, concentration of mineral elements), the concentration of the substance to be tested, and the concentration of the bacterial inoculum. While it is possible to reproduce the first two parameters in biodegradation tests, the inoculum represents a significant challenge for the reproducibility of test results. Unfortunately, microbial inocula used in OECD biodegradation tests are one of the most poorly controlled variables. High variability in the biodegradability determination of chemicals in OECD tests is potentially due to the characteristics of the inocula (presence or absence of biodegraders, adaptation to the chemical, cell density, and presence of protozoa) which control both lag phase and biodegradation kinetics, which may lead to problematic interpretation (Thouand *et al.*, 1995). Tested inocula should be characterized in terms of their size and biodegradation potential. Unfortunately, bacterial density estimation methods are rarely applied and so far there are no practical methods/protocols to measure inoculum diversity in the industrial laboratories (van Ginkel *et al.*, 1995; Thouand *et al.*, 2011). For instance, in

screening tests maximum inoculum size used (amounts of activated sludge or secondary effluent per litre test medium) is estimated based on the oxygen consumption, due to the metabolic activity of the inoculum (Struijs *et al.*, 1995). Therefore, there is a need for standardized protocols which should be used before biodegradation testing to determine the overall size of inoculum, while application of advanced techniques would be useful to determine inoculum quality (number of specific degraders) and the diversity of functional communities involved in biodegradation processes.

OECD screening tests can involve variable cellular density in tested inocula, for example in activated sludge and river water the number of specific degraders may vary from 3.7×10^6 to 2.5×10^4 cells/L, respectively (Thouand *et al.*, 1995, Thouand *et al.*, 2011). The modified French Association for Standardization (AFNOR) method specified use of 5×10^5 cells/mL in test assays and the modified Sturm test suggested 10^4 - 2×10^5 cfu/mL (Painter, 1995). It was reported by Thouand *et al.* (1996) that only 10^5 total cells/L of degraders are needed for a 99.9% chance of a positive response in *para*-nitrophenol biodegradability tests. Inocula used in simulation tests can vary. Surface water (e.g. from estuary or marine water), sediment or soil samples support different numbers of bacteria, and have variable community composition and diversity. The influence of the inoculum on the test is due to its origin but also its concentration of live bacteria (Thouand *et al.*, 1995) the adaptation of bacteria to the test molecule (Barkay and Pritchard, 1988), and the presence of protozoa (Ramadan *et al.*, 1990). Ramadan *et al.*, (1990) reported that lower cell density of degraders introduced into large volumes of chemically polluted water or soils may lead to lack of proliferation of degraders prior to biodegradation. This could reflect competition for the limited supply of inorganic nutrients or predation of introduced population of bacteria before it becomes sufficiently large to biodegrade the chemical. Nishino and Spain (1993) showed cell density dependent adaptation of *Pseudomonas putida* to biodegradation of *para*-nitrophenol (PNP) and they observed a delay of PNP degradation at a low cell density. Ingerslev *et al.*, (2000) also investigated the effect of low bacterial numbers on biodegradation results. They reported that the length of the lag phase tended to increase with decreasing inoculum concentration, with high bacterial concentrations increasing the probability of a successful biodegradability test. It is important to control the number of specific degraders used in

biodegradation tests in order to limit the risk of errors in biodegradability tests which are mainly associated with contradictory test results when testing the same chemical using inocula with variable numbers of specific degraders (Thouand *et al.*, 1996).

Increasing bacterial concentration could lead to carbon contamination and Thouand *et al.* (1995) pointed out that this attempt should be limited since (1) test chemical should be the only source of carbon and energy for bacteria to limit cometabolism and biodegradation rate modification and (2) biodegradability measured using indirect methods such as organic carbon disappearance, oxygen consumption or CO₂ production are accurate only if background carbon consumption is low. Thouand *et al.* (2011) suggested that carbon contamination could be reduced by washing of concentrated inoculum followed by centrifugation. Therefore, strategies have been developed to improve the size and quality of test inocula. Adaptation of tested inocula is a more environmentally realistic approach for increasing the probability of chemical biodegradation since in the natural environments bacterial communities are continuously exposed to a variety of chemicals. The inoculum adaptation decreases the risk of error in biodegradability tests in comparison with non-adapted inocula, since it does not change the number of biodegraders in comparison with number of total or cultivable bacteria, and the carbon contamination is reduced (Thouand *et al.*, 1996). At the same time a long preculture process could be responsible for false negative test results due to the circumstance that used inoculum has a limited lifetime and may deteriorate in time and lose its specific degradation ability (Toräng and Nyholm, 2005; Vázquez-Rodríguez *et al.*, 2011). Therefore, other approaches to improve biodegradability testing have been developed. Semi-continuous pre-exposure procedure (SCEP) was suggested by Toräng and Nyholm (2005) for inoculum preadaptation prior to biodegradation tests. They recommended this system as an option in biodegradability simulation studies with low chemical concentrations if adaptation takes place slowly or unpredictably. For example, problems with the consistency of results was observed for some recalcitrant chemicals (4-chloroaniline, maleic hydrazide and pentachlorophenol) in surface water simulation tests, where long and variable lag phases were reported as well as failed chemical biodegradation. Testing those chemicals required the SCEP approach to improve prediction of chemical biodegradation (Ingerslev and Nyholm, 2000).

Biodegradability tests may involve inoculum from different origins such as: river water, sea water, activated sludge, soil (Thouand *et al.*, 2011), which also should be taken into account while running tests since the inoculum diversity is related to its origin. For example, Forney *et al.* (2001) demonstrated that the diversity of several activated sludges differed not only worldwide but also changed in the same wastewater treatment plant during the course of one working day. Moreover, specific degraders for non-readily degradable substances are more likely to be found in the sediment of rivers and coastal areas than in the water, whereas samples from pristine areas contain very small numbers of specific degraders (Thouand *et al.*, 2011). Franklin *et al.* (1999) reported higher cell numbers and microbial diversity in contaminated zones of ground water than in pristine areas. Additionally, pristine aquifers are carbon-limited environments, with bacterial populations in a state of starvation and reduced activity (Goldscheider *et al.*, 2006). An inoculum with high species diversity has a greater chance to contain chemical-degrading species, and therefore, to degrade test chemicals in comparison with less diverse inoculum. Margesin *et al.* (2003) reported that contaminated environments harbour a wide range of chemical-degrading microorganisms, which play crucial role in the bioremediation processes.

1.6.3.1. Biofilm as inoculum

Stream biofilms are complex communities composed of algae, cyanobacteria and bacteria embedded in a polysaccharide matrix attached to substrates such as rocks, sediments and aquatic plant surfaces (Vinten *et al.*, 2011). Biofilm accumulation in riverbeds or on suspended particles contributes largely to the removal of contaminants from the water, and thus the biofilm enhances self-purification of surface water (Muia *et al.*, 2003; Edwards *et al.*, 1990). In rivers and streams, biofilms are the first to interact with dissolved substances such as nutrients, organic matter, and chemicals. Biofilms integrate the effects of environmental conditions over extended periods of time, mainly because of their rapid growth, species richness, and the physiological variety of the organisms of which they are formed. They have been used for routine monitoring and could be useful as “early warning systems” of ecosystem disturbances (Sabater *et al.*, 2007). Brümmer *et al.*, (2000)

observed stability of river biofilm community structure in spite of significant variation in chemical and biological parameters during the year. Standardized ways of obtaining biofilm samples from natural ecosystems could be applied in biodegradation testing where a biofilm is used as an inoculum source. Araya *et al.* (2003) compared the biodegradation capacity of biofilms sampled from a natural river located in an industrial area through the use of a river die-away biodegradation test which employs sterilized river water (circulated by submersible pumps) and follows the disappearance of an added amount of test compound. They illustrated the important contribution of natural river biofilm communities to the river die-away test conducted under laboratory conditions. Biofilms had a significant participation in the biodegradation capacity of diazinon-contaminated river ecosystems (Tien *et al.*, 2011). Natural river biofilms developed on ceramic disks showed 99.9% removal of diazinon at initial concentration of 5 mg/L, over 26 days, in the laboratory tests. Since biodegradation of many groups of chemicals requires relatively rare species to be present in the tested inoculum (Thouand *et al.*, 2011) biofilms have potential as an inoculum since they consist of complex bacterial communities, which represent natural microbial populations.

Studying microbial community composition within biofilms, its diversity and changes after exposure to organic chemicals is crucial to understand the effects of chemicals on aquatic ecosystem. For instance, Yergeau *et al.* (2012) determined the active community composition within river biofilms and subtle changes in microbial communities were detected after short-term exposure to low concentrations (0.5-1.0 µg/L) of antibiotics: erythromycin, sulfamethoxazole and sulfamethazine. Profile of river biofilm microbial community reflects the state of natural ecosystems (Yergeau *et al.*, 2010) and reveals the ecological responses of biofilms to stream water quality (Vinten *et al.*, 2011). Functional shifts in bacterial communities could be responsible for a disruption of important ecosystem processes, and therefore, detection of such shifts is important for monitoring effects of chemicals on river ecosystems which is crucial for chemical risk assessment.

1.6.4. Bioavailability

Knowledge of the processes involved in distributing chemicals among ecosystem compartments is essential to understand their behaviour in the environment (Rubio *et al.*, 1996). Sorption, partitioning from water to sediment, desorption and chemical aging may affect chemicals concentration, distribution in environmental compartments and bioavailability to microorganisms involved in biodegradation (Hatzinger and Alexander, 1995; Rice *et al.*, 2004; Ying, 2006). Microorganisms developed several strategies to improve bioavailability of chemicals in the environment. Bacteria can enhance diffusive flux of poorly available chemicals by shortening of the diffusion distance by attachment to the substrate, enhancing the dispersion and solubility of chemical by biosurfactant production, and reducing the cell surface concentration due to high substrate affinity (Wick *et al.*, 2001).

Sorption may lead to increased persistence of chemicals in environmental compartments (Rubio *et al.*, 1996; Ying, 2006). In aquatic systems, partitioning of nonpolar class of compounds plays a key role in transfer between environmental compartments. The sediment/water partitioning depends on the carbon content, carbon partition coefficient (K_{oc}), diffusion, and bioturbation processes (Chevreuil *et al.*, 1998). Low water solubility of some chemicals means that they pass very slowly from a non-aqueous to the aqueous phase, where they are metabolised by microorganisms (Bardi *et al.*, 2000). Studies showed that the presence of sediment would lead to a higher biodegradation rate because sorbed contaminants could be degraded by solid-phase bacteria (Xia *et al.*, 2006). Sorption of phenanthrene to river sediments was reported by Cornelissen and Gustafsson (2004) and distribution of considerable number of pharmaceuticals in rivers depends on sorption to suspended solids and sediment (e.g. famotidine, salbutamol) (Silva *et al.*, 2011). Surfactants also represent a group of chemicals which are easily sorbed to sediment and soil (Ying, 2006).

A significant proportion of pharmaceuticals and some chemicals used in HPC are ionisable substances, which should be considered while studying their removal during wastewater treatment (Fu *et al.*, 2009). The physico-chemical properties of chemicals affect their removal performance in WWTPs. Several neutral and acidic pharmaceuticals were detected in Canadian effluents from WWTPs (Metcalf *et al.*,

2003). Hydrophilic medicines (e.g. sulfonamids) appear to be quickly transported to surface waters, whereas the transport of hydrophobic compounds is much slower, with concentrations measured in drainage outfalls many months after veterinary medicines application (Boxall *et al.*, 2003). Kolpin *et al.* (2002) reported a range of organic wastewater contaminants, representing residential, industrial and agricultural origins, which were detected in 80% of the streams sampled. Also, occurrence of organic wastewater compounds e.g. 3-beta coprostanol, 4-nonylphenol, beta-sitosterol in an effluent-dominated stream in Northeastern Kansas was reported by Lee and Rasmussen (2006). Chemicals that accumulate in environmental compartments may be persistent and not readily biodegradable due to their low susceptibility to biodegradation and high sorptive properties. At the same time, some chemicals (e.g. surfactants) can enhance solubilization and removal of contaminants. For example, LAS, which has been found to increase water solubility of chemicals, increased leaching of polychlorinated dibenzo-*p*-dioxins and dibenzofurans (PCDD/Fs) measured in soil and ash (Schramm *et al.*, 1995). Such surfactants increase aqueous solubility of compounds and make them available for catabolism (Bardi *et al.*, 2000). Sorption of some chemicals to soil can also increase with time, demonstrating an aging effect (Rice *et al.*, 2004). Several mechanisms have been described for the aging of chemicals in soils and sediments. Due to reactions that alter the structure of the molecule (e.g. polymerization or covalent binding to humic substances) a desorption-resistant fraction is created, which becomes entrapped in the soil or sediment (Hatzinger and Alexander, 1995).

1.6.5. Test substance concentration

There is a growing focus on the problem of extrapolation of biodegradability data obtained from standard laboratory tests to environmental conditions observed in field. OECD tests assess rates of biodegradation at chemical concentrations far in excess of those likely to be found in natural ecosystems (Lapertot and Pulgarin, 2006). Concentration of chemicals in the WWTPs or in the environment are usually in the range of ng/L to µg/L compared to 10-400 mg carbon per litre in most screening tests (Ahtiainen *et al.*, 2003; Kolpin *et al.*, 2002). However, simulation test for biodegradation of chemicals in surface water systems provide site-specific

information about biodegradation rates, and adaptation behaviour at environmentally realistic concentrations (at least below 100 µg/L and normally at or below 10 µg/L) (Comber and Holt, 2010). Extrapolations using data from tests at high concentrations may not reflect what takes place at low concentrations (Lapertot and Pulgarin, 2006). Toräng *et al.* (2003) demonstrated that direct use of the biodegradation rate data obtained for mecoprop and 2,4-dichlorophenoxyacetic acid at high (>100 µg/L) concentration in aerobic aquifer, may grossly overestimate the actual degradation rates at environmental relevant concentrations in aquifers (in the order of 0.1 µg/L). Concentration of the compound may be a significant factor affecting its behaviour in the environment, and therefore, its susceptibility to microbial attack (Efroymson and Alexander, 1995). Chemicals at low concentrations do not serve as primary substrates but are more likely to be degraded as secondary substrates (non growth substrates) concurrently with a variety of naturally occurring carbonaceous compounds (Berg and Nyholm, 1996). Organic substrates present in those environments might be preferentially degraded or co-metabolized with the primary contaminant resulting in lower biodegradation rates. For example, the effect of organic carbon supplementation on the biodegradation of ionic liquids in sewage sludge treatment was reported by Markiewicz *et al.* (2011). It was found that the presence of organic carbon (e.g. presence of chemicals) diminished the rate of biodegradation. Some evidence exist that nutrients or other compounds also alter the rates of transformation by heterotrophic microorganisms. For example, inorganic nutrients amendment stimulated mineralization of higher concentrations of herbicide mecoprop in soil and aquifer sediment (de Liphay *et al.*, 2007). On the other hand, presence of some chemicals may cause an inhibitory effect on microbial populations. Stasinakis *et al.* (2008) reported the toxic effect of triclosan (TCS) and nonylphenol (NP) on activated sludge heterotrophic and autotrophic microorganisms, and a possible risk for deterioration of nitrification in activated sludge systems due to the presence of TCS. Differences in biodegradation behaviour between the two concentration levels of aniline, 4-chloroaniline and pentachlorophenol were described by Berg and Nyholm (1996). They found that percentage of test compound removed by unadapted sludge had a tendency to be higher with test compound dosed at trace concentrations (10 µg/L) than at standard (high) concentrations (20 mg DOC/L). By contrast, degradation of other compounds (halobenzoate, nitroacetic

acid, 2,4-dichlorophenol and isopropyl N-phenylcarbamate) was inhibited at low concentrations (Pitoy *et al.*, 2011).

Extents and rates of adaptation, important for biodegradation behaviour, can also be greatly influenced by the chemical concentration. Low concentrations can be expected to prolong adaptation in comparison with higher concentrations (Berg and Nyholm, 1996). As a result many organic compounds persist in the environment. The reason for this could be that the time is needed for the population of degraders to become sufficiently large or active to bring about chemical biodegradation or for microorganisms to synthesise and/or activate the required enzymes for biodegradation (Grady, 1985). In some studies concentration thresholds have been reported below which no adaptation could be detected. Efroymson and Alexander (1995) reported lack of phenanthrene mineralization in a mixed culture at concentrations of 0.6–20 µg/L. However it was found that some organic compounds are mineralized even at ng/L levels, which suggests that some chemicals can be transformed at trace levels. Degradation of N-nitrosomorpholine (NMOR) was reported by Pitoy *et al.* (2011) at 200 ng/L and no threshold effect of this concentration was found.

Concentration of test compound should be considered in biodegradation tests since it affects chemical behaviour in test system and biodegradation mechanisms. Although it is challenging to measure environmentally realistic concentrations of tested chemicals in complex matrices of environmental samples, efforts should be made to study chemical biodegradation at low concentrations to achieve more accurate prediction of their fate in the environment.

1.6.6. Kinetics

Precise prediction of chemical concentration in the environment depends on the knowledge of degradation rate constants for chemical in the various environmental compartments. Controversy exists on the type of kinetic model needed to depict mineralization rates of microbial communities in natural ecosystems (Painter, 1995). In screening tests the first-order kinetic model can be used instead of 10-day window

to describe degradation kinetics (e.g. respirometric biodegradation test based on OECD 301). However, the first-order rate constant may vary for different industrial chemicals (e.g. benzene, methyl parathion, 4-chlorophenol) due to the quantity and quality of biomass used (Reuschenbach *et al.*, 2003). In simulation tests kinetics are established based on the concentration of test substance used. Therefore, concentrations applied should be low enough to avoid incorrect estimation of rate constants (Nyholm *et al.*, 1996). First-order kinetics were reported for degradation of some chlorinated and nonchlorinated aromatic compounds e.g. 1,2,4-trichlorobenzene (Rapp, 2001), mecoprop and 2,4-dichlorophenoxyacetic acid (Toräng *et al.*, 2003) at low concentrations. Shifts in biodegradation kinetics of mecoprop and 2,4-dichlorophenoxyacetic acid were observed by Toräng *et al.* (2003). First-order non growth kinetics were determined at low herbicide concentrations, while growth of biomass was observed along with biodegradation at higher herbicide concentration. It has been found that in practice, especially in waste water treatment, the mathematical model for the growth of microorganisms Monod (empirical) and Michaelis-Menten (theoretical) equations are not always obeyed. For example, only a minority of active bacterial cells present in activated sludge may divide within the system and the majority inactive bacteria may metabolize substrates without dividing (Painter, 1995). The chemical biodegradation follows Monod kinetics when the bacterial growth is occurring as the substrate disappears. Although, the Monod equation is widely accepted, there are certain limitations in applying it to biodegradation in the aquatic environment. For instance, there is a lack of consideration of other factors which affect biodegradation in the environment, such as the presence of other substrates, predation by protozoa and phage, toxic chemicals produced by other organisms and the availability of inorganic nutrients (Painter, 1995).

1.7. Microbial ecology in biodegradation testing

Identification and characterization of microbial communities isolated from contaminated environments carried out using the culture-dependent methods limited species detection to the culturable ones. Culturability in nearly all environments

varies from 1 – 10% (Porter *et al.*, 2004), because it is difficult to simulate complex environmental conditions essential for proliferation of specific groups of bacteria. For example, more than 99.9% of the natural bacterioplankton community in sea water could not be cultivated using marine agar (Eilers *et al.*, 2000). The application of culture independent methods such as molecular techniques, enable the detection of larger numbers of previously unrecognized microorganisms as well as complex microbial diversity in contaminated sites. Culture-independent approaches can be applied for characterisation of inoculum used for biodegradation testing, which is crucial for reproducibility of tests and understanding processes, which take place in natural environment. Therefore, microbial ecology methods such as: Terminal Restriction Fragment Length Polymorphism (TRFLP), Quantitative Polymerase Chain Reaction (QPCR) and functional gene assays, are useful techniques that identify and quantify microbial population and catabolic genes involved in biodegradation. For example Paul *et al.* (2006) used TRFLP for profiling bacterial community structure and its changes, which occurred at a pesticide-contaminated site during bioremediation. Functional gene approaches can be used to study the diversity of specific degraders. Ní Chandhain *et al.* (2006) determined microbial dioxygenase gene shifts during polycyclic aromatic hydrocarbon (PAH) biodegradation. Changes of key catabolic genes (catechol 2,3-dioxygenase, catechol 1,2-dioxygenase and alkane-catabolic genes (*alk*)) and microbial community structure were studied by Zhang *et al.* (2008) during the degradation of nonylphenol ethoxylates (NPEOs) and NP in natural water microcosms. Number of copies of functional genes involved in the biodegradation pathways of chemicals, measured in environmental samples, can indicate the presence of specific degraders, and therefore, could be used to determine the potential of environmental compartments to biodegrade chemicals. Overall, microbial ecology methods are important tools for better understanding of processes (e.g. shifts in bacterial community structure, proliferation or disappearance of specific degraders) associated with the biodegradation of contaminants in natural environments. A mechanistic understanding of bacteria mediating biodegradation processes may be useful for better test design, data modeling and more accurate interpretation of biodegradation test results.

1.8. Future perspectives

Culture-independent approaches used in microbial ecology are mainly applied to conduct microbial community profiling and are a preliminary steps before the more advanced (e.g. metagenomic, metatranscriptomic) analysis is performed. Metagenomics and metatranscriptomics are advanced methods since they allow to study genetic material recovered directly from environmental samples, while traditional microbiology and genomics rely upon cultivated microbial cultures (Desai *et al.*, 2010). High-throughput technologies from the genomics, metagenomics, proteomics and metabolomics are the most promising tools to understand the biodegradation processes in depth (Trigo *et al.*, 2009). Application of “omics” gives not only the opportunity to confirm the processes/mechanisms occurring in the environment, but also to answer questions arising during the environmental studies (e.g. explanation of test failures, detection of new degraders, characterization of functional genes and possible biodegradation pathways). A variety of emerging high-throughput technologies have proved to be useful for studying biodegradation processes in the environment since they link microbial phylogeny to its ecological function. The potential application of these techniques (**Table 1.5**) for development of new bioremediation strategies of contaminated sites was overviewed by Stenuit *et al.* (2008). Advanced high throughput techniques like 454 sequencing enable fast and in depth screening of environmental samples for specific degraders and functional genes involved in biodegradation pathways of chemicals. In depth analysis of microbial consortia present in environmental compartments is useful for studying their function, community structure and how they are affected by different factors. Information obtained through application of such techniques is essential for better understanding of microbial networks present in natural ecosystems since it allows specific functions to be assigned to certain groups of microbes. ‘Omics’ enable transition between the environment and laboratory tests, and vice versa, which is crucial for extrapolating results into the environment, and therefore, for better biodegradation assessment under environmentally realistic conditions.

Table 1.5: Application and potential of high-throughput technologies in biodegradation studies.
Source: Stenuit *et al.*, 2008

Technique	Typical target molecule	Application	Potential
Fingerprinting techniques, phylogenetic oligonucleotide arrays (PAOs), community genome arrays (CGAs), real-time Polymerase Chain Reaction (QPCR)	DNA RNA	<ul style="list-style-type: none"> Community structure and dynamics 	Relate microbial community analysis to the metabolic function of specific groups of bacteria
Metatranscriptomics	RNA		Identification of the
Metaproteomics	Proteins	<ul style="list-style-type: none"> Community function 	biodegradation potential and the function of specific microbial communities
Metabolomics	Metabolites		
Biosensors	Proteins Metabolites	<ul style="list-style-type: none"> Presence of undefined chemicals Nature and concentration of the chemicals Toxicity of the chemicals for living organisms Decrease in chemical concentration, and/or transformation 	Monitoring “real-time” the performance of a bioremediation process
Cultivation (+ genome sequencing), Metagenomics	DNA	<ul style="list-style-type: none"> Use of exogenous biocatalysts Search for new catabolic activities 	New metabolic functions of microbial communities

1.8.1. Metagenomics

Recent advances in metagenomics in which collective microbial genomes are sequenced and screened for functional genes and phylogenetic markers provide an opportunity for linking microbial diversity with function (Malik *et al.*, 2008). The recent development of ultra-high throughput sequencing technologies do not require cloning or PCR amplification, and can produce huge number of DNA reads at an affordable cost. The analysis of such datasets aims to determine and compare the biological diversity and the functional activity of different microbial communities (Huson *et al.*, 2009). For example Mackelprang *et al.* (2011) reported that metagenomics allowed examination of whole biochemical pathways and associated processes, as opposed to individual pieces of the metabolic matrix. Their permafrost metagenome analyses revealed changes in many microbial, phylogenetic and functional gene abundances and pathways they studied which were caused by thaw of permafrost. Development of metagenomic arrays for uncultured microorganisms from contaminated environments can greatly improve our understanding of microbial interaction and metabolism to facilitate the development of suitable bioremediation strategies for environment clean up (Malik *et al.*, 2008).

1.8.2. Metatranscriptomics

Another approach for studying bacterial communities is metatranscriptomics, which refers to the analysis of the collective transcriptomes of a given habitat. It is based on direct retrieval and analysis of microbial transcripts from environmental samples, where environmental mRNA obtained from total RNA is reverse transcribed, amplified and used for analysis (Stenuit *et al.*, 2008). For example, transcriptomic analysis of a marine bacterial community enriched with dimethylsulfoniopropionate (DMSP) was reported by Vila-Costa *et al.* (2010). They observed increased abundance of transcripts for *Gammaproteobacteria* and *Bacteroidetes* as well as overexpression of genes involved in the biodegradation of C3 compounds after DMSP addition.

1.8.3. Metaproteomics

Metaproteomics is the study of the entire protein content of a given habitat, and it has greater potential than genomics for the functional analysis of microbial communities. It is known that mRNA expression levels (the transcriptome) may be unreliable indicators of the abundance of the corresponding proteins. In metaproteomics, complex mixtures of proteins from an environmental sample are separated and fractions of interest are analyzed by high-throughput mass spectrometry-based analytical platforms (Stenuit *et al.*, 2008). Exploring the differential expression of a wide variety of proteins and screening of the entire genome for proteins that interact with particular mineralization regulatory factors is helpful to get insights into biodegradation (Vieites *et al.*, 2009). Proteomics also plays an essential role in determining the physiological changes of microorganisms under specific environmental influences, while functional proteomics would predict metabolism of contaminants by degrading organisms (Chauhan and Jain, 2010).

1.8.4. Systems biology approach to biodegradation

Microbial interactions are essential for biodegradation processes in natural environments. General knowledge about the processes operating within microbial community is not sufficient enough to determine the role of individual members and their interactions within the community. For biodegradation studies it is crucial to understand how those relationships could be affected by the environment and emerging chemicals, and vice versa (Zengler and Palsson, 2012). Also, the biodegradation processes are framed in a complex web of metabolic and regulatory interactions which are extremely difficult to approach with the traditional molecular methods. For a long time experimental procedures only allowed the analysis of a few enzymes at a time. Therefore, new techniques and in particular large-scale approaches were developed. They use the system, which is divided, into parts to study them individually but also it is possible to look into interactions between the parts and how they influence each other. The recent accumulation of knowledge about the biochemistry and genetics of the biodegradation process, and creation of

structured databases, has opened the door to systems biology. It has been set up to examine complex biological interactions and processes, but it also allows gathering of more information about the biodegradation process in the real environment where it takes place (Trigo *et al.*, 2009).

1.8.5. Advantages and disadvantages of high throughput methods

Emergence of specialized techniques for studying the microbial genome, transcriptome, proteome, metabolome enabled development and successful execution of bioremediation processes (Desai *et al.*, 2010). Recent molecular and “omics” approaches enable exploration and better understanding of microbial biodegradation processes (**Figure 1.4**), and therefore, improvement of chemical risk assessment in environmental compartments. This will lead to development and application of new, more effective strategies to sustain a cleaner environment.

Although, high throughput techniques are emerging tools in biodegradation studies they are still relatively new techniques. One of the biggest disadvantages of “omics” is their blind application. While there is a place for discovery science using high-throughput techniques progress is usually achieved in studies that are applied with a clear hypothesis in mind. The major concerns arising around “omics” are also linked with constant development of technologies and urge to bring new improvements and applications by companies such as Roche or Illumina. This requires constant development and education of scientists in order to be on top of those approaches. Another issue is the interpretation and management of massive amount of data, which is generated by high throughput technologies. Therefore, development of efficient statistical algorithms, pipelines and other bioinformatic tools is crucial to obtain the good quality data and to perform further analysis. Moreover, the costs of generating high throughput data and data analysis are still high and not always affordable. Hopefully, in the near future, decrease in the costs of such analysis will increase the availability of these techniques and their applicability for biodegradation studies (Shendure and Ji, 2008; Desai *et al.*, 2010).

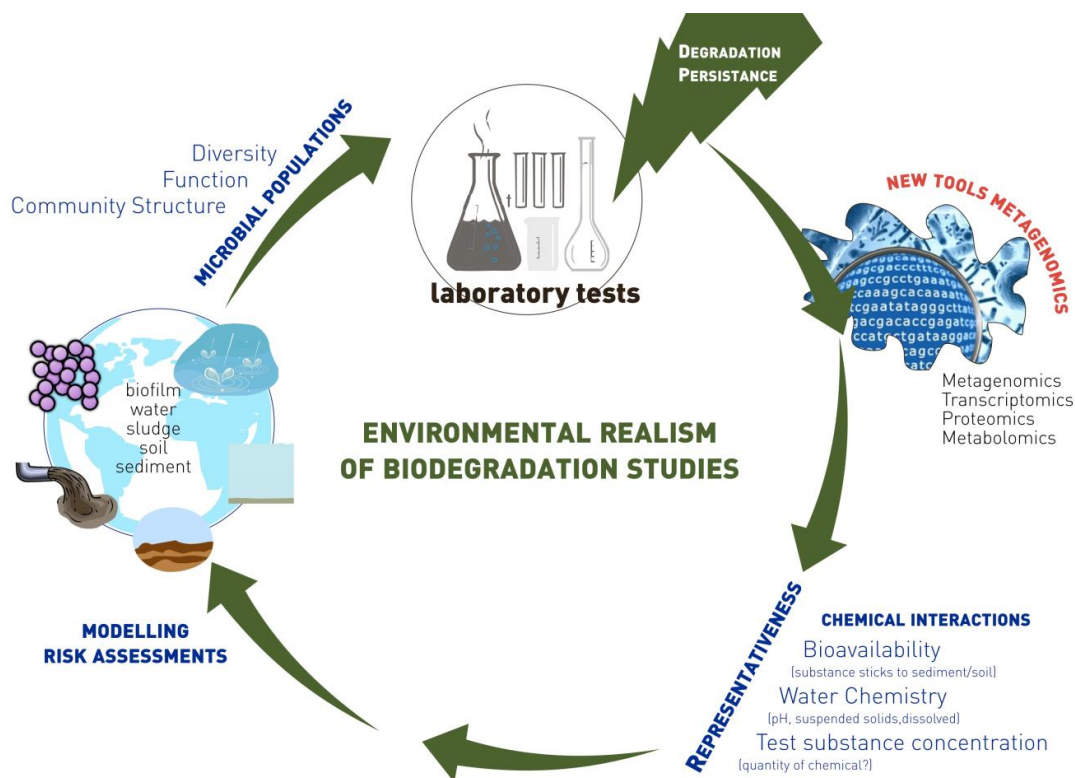


Figure 1.4: Schematic of an approach to study effects of environmental realism on biodegradation.

1.9. *Para*-nitrophenol (PNP)

The choice of a suitable model compound for biodegradation tests depends on its solubility in water, resistance to microbial biodegradation, and potential environmental impacts. Although *para*-nitrophenol (PNP) does not belong to DTD chemicals, it is used here as a model compound to conduct experiments which address general questions regarding chemical biodegradation in the environment to later inform specific studies. PNP is a good model compound since it is present in the environment, it has been used in biodegradation tests and there is available information on pathways and functional genes involved in PNP biodegradation.

1.9.1. PNP in the environment

Nitroaromatic compounds, including nitrophenols, are widely distributed in environment, and among these chemicals, *para*-nitrophenol (PNP) (**Figure 1.5 a** and **1.5 b**) is one of the most important both in terms of quantities used and potential

environmental impacts (ATSDR, 1992; Qiu *et al.*, 2007). It is a manufactured chemical which does not occur naturally in the environment and the primary anthropogenic source of PNP in the air is probably industrial manufacturers. Very little of this compound is directly released to surface water or soil. Like other nitroaromatic compounds, it can be formed as a result of atmospheric photochemical reactions of several aromatic compounds from anthropogenic sources (ATSDR, 1992; Calza *et al.*, 2008). It can be also found in vehicular exhausts as a result of the thermal reaction of fuel with oxides and nitrogen. Additionally, PNP is formed as a degradation product and it is an impurity in parathion formulation (ATSDR, 1992).

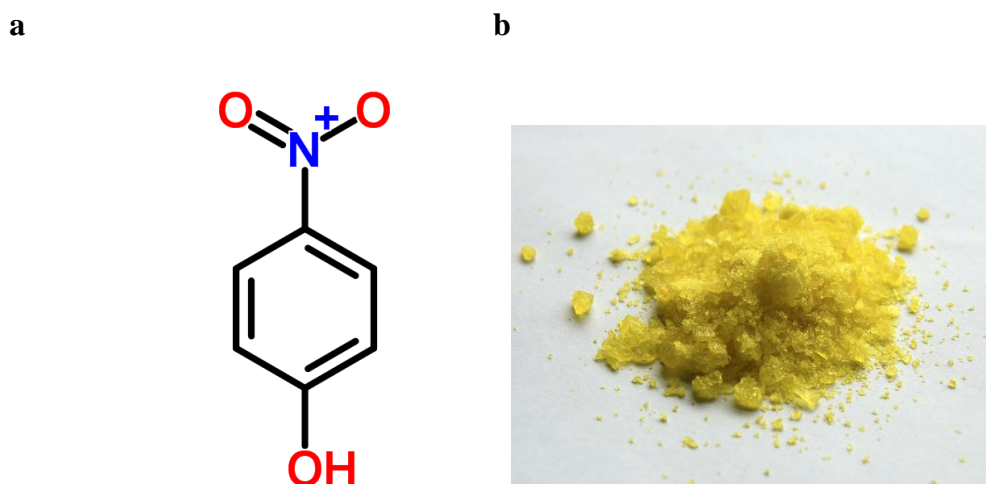


Figure 1.5: *Para*-nitrophenol.

Where: a-Chemical structure of *para*-nitrophenol, source: ChemSpider, 2012; b-physical appearance of *para*-nitrophenol, source: Wikipedia, 2012 a.

1.9.2. Application and properties of *para*-nitrophenol

PNP has application in agriculture, dyes, pigments, engineering polymers and pharmaceuticals. It is also used as fungicide for leather, production of parathion and other organic synthesis (Montgomery and Welkon, 1955; Cho *et al.*, 2000; Qiu *et al.*, 2007). PNP is a major urinary metabolite of parathion and can be used as a biomarker of human exposure to pesticides since it is a breakdown product of parathion and fluoridifen (Rogers and Emon, 1993). PNP has a good solubility in water (**Table 1.6**), the log K_{ow} of 1.9 and vapor pressure of 9.86×10^{-2} Pa (Comber and Holt, 2010). It is toxic to plant, animal and human health, with mutagenic and carcinogenic activities. The purification of wastewaters contaminated with nitroaromatic chemicals is very difficult since they are resistant to conventional

treatment techniques (Kuşçu and Sponza, 2009). In air, destruction of PNP is mainly due to photolysis and physical removal processes such as gravitational settling of aerosols and wet deposition by rain and snow. In water, both photolysis and biodegradation take part in removal of PNP; photolysis is important in near surface water. In soil the biodegradation of PNP is the most important fate process (ATSDR, 1992).

Table 1.6: Physico-chemical properties of *para*-nitrophenol.

Source: Wikipedia, 2012 a.

Properties	
Molecular formula	$C_6H_5NO_3$
Molar mass	139.11 g/mol
Appearance	Colorless or yellow pillars
Melting point	113–114 °C
Boiling point	279 °C, 552 K, 534 °F
Dissociation constant	pKa 7.16 (22 °C)
Solubility in water	10 g/L (15 °C)
	11.6 g/L (20 °C)
	16 g/L (25 °C)

1.9.3. *Para*-nitrophenol as a model compound

PNP has been used as a model compound for chemical biodegradation studies by several authors (Ingerslev *et al.*, 2000; Nyholm *et al.*, 1996, Nyholm *et al.*, 1992; Thouand *et al.*, 1995) with variable mineralization results. The biodegradation of PNP can be rapid, but sometimes it occurs after a long lag phase. Toräng and Nyholm (2005) considered *para*-nitrophenol to be readily biodegradable after a lag phase. At 100 µg/L in natural surface waters they reported that the lag phase was reduced from 10 days to <1 day following an adaptation period of between one and five weeks. PNP is also used by Davenport *et al.*, (2009) as a reference standard in their studies to investigate the importance of microbial density and diversity of inocula used in ready tests. Initial results indicated a high probability of

biodegradation (>70% parent compound degradation) with enhanced inocula concentrations and extended test duration (60 days) with low variability between inocula from six different locations for activated sludge and river water. Greater variation in the biodegradation of PNP was observed when a 28-day test was used. However, PNP is recommended as a reference chemical for the modified SCAS test (OECD 302A) (OECD, 1981b). It is also biodegradable in enhanced tests with increased biomass. Measured data (n=18) in non-standard tests suggest a median half-life for PNP of 2.5 days in the freshwater environment with a range from 1.3-77 days (Comber and Holt, 2010).

1.9.4. Biodegradation pathways and functional genes

There have been many reports on the biodegradation of PNP (Munnecke and Hsieh, 1974; Spain and Gibson, 1991; Roldán *et al.*, 1998; Lima *et al.*, 2003; Kulkarni and Chaudhari, 2006; Qiu *et al.*, 2007). PNP biodegradation pathways, including genes encoding key enzymes involved in its biodegradation, are also well characterized (Kitagawa *et al.*, 2004; Perry and Zylstra, 2007; Takeo *et al.*, 2008; Zhang *et al.*, 2009). Many PNP degrading bacterial strains belonging to the genera *Flavobacterium*, *Pseudomonas*, *Moraxella*, *Nocardia*, and *Arthrobacter* have been isolated and their metabolic activity on PNP was defined. Two major degradation pathways of PNP (**Figure 1.6**) have been characterized (Chauhan *et al.*, 2010; Kitagawa *et al.*, 2004). Both pathways lead to degradation of PNP to maleylacetate but differ in the initial steps. PNP biodegradation via hydroquinone (hydroquinone pathway) has been found in Gram-negative bacteria such as *Burkholderia* spp., and *Moraxella* spp. (Prakash *et al.*, 1996; Spain and Gibson, 1991), whereas PNP biodegradation via 4-nitrocatechol (4-nitrocatechol pathway) has been found in Gram-positive bacteria such as *Bacillus* spp., and *Arthrobacter* spp. (Jain *et al.*, 1994; Kadiyala and Spain, 1998).

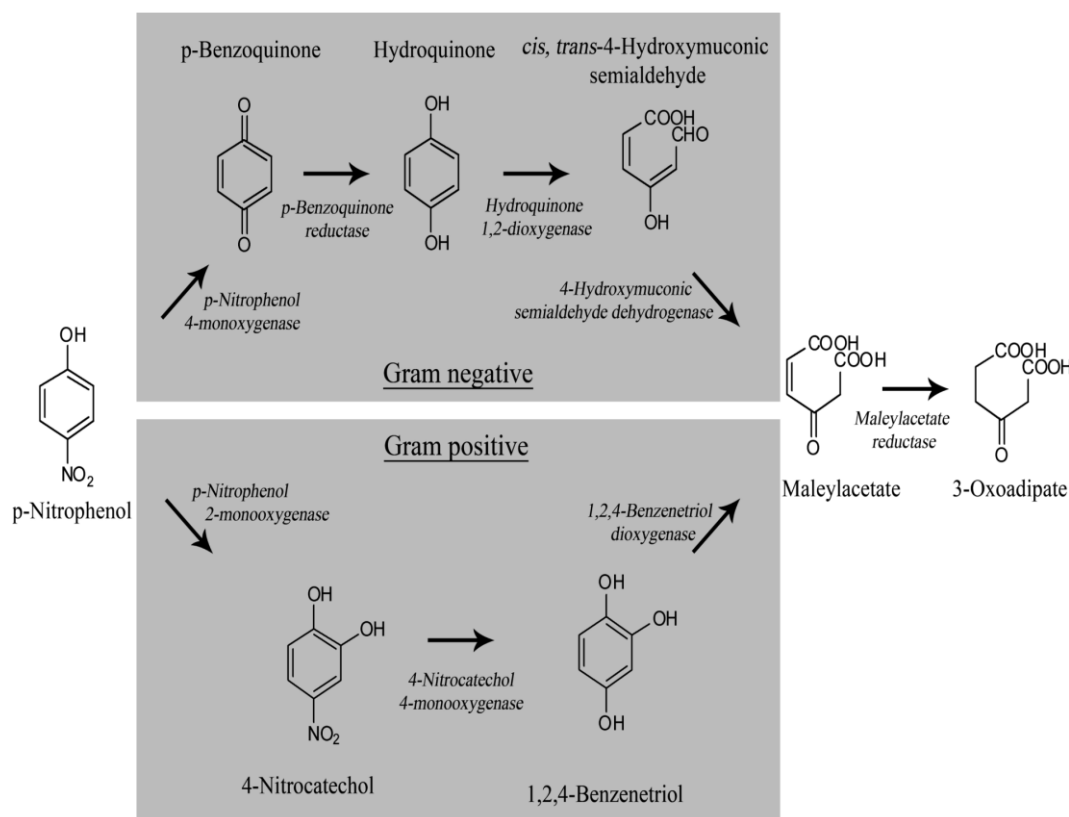


Figure 1.6: Pathways for PNP degradation in bacteria.

Source: Chauhan *et al.*, 2010.

PNP degradative genes *pnpA* and *npdA2* are responsible for the initial reaction in PNP biodegradation pathways in Gram-negative and Gram-positive bacteria, respectively. Gene *pnpA* encodes *para*-nitrophenol 4-monoxygenase (PnpA), which degrades PNP into hydroquinone through *para*-benzoquinone, and *npdA2* encodes *para*-nitrophenol 2-monoxygenase (NpdA2), which degrades PNP into 4-nitrocatechol.

Maleylacetate reductase (MAR) is a part of both PNP degradation pathways (**Figure 1.6**) and is responsible for the NADH- or NADPH-dependent reduction of maleylacetate to 3-oxoadipate or substituted maleylacetates to substituted 3-oxoadipates (Seibert *et al.*, 2004). Maleylacetate is also an intermediate in the biodegradation of a number of other chemicals (**Figure 1.7**) and generally leads to production of 3-oxoadipate which is converted to succinyl-CoA and acetyl-CoA (Harwood and Parales, 1996), which enter the tricarboxylic acid cycle (TCA). Maleylacetate reductase genes have been detected and described in many bacteria e.g. in *Rhodococcus opacus* 1CP (gene *macA*; Seibert *et al.*, 1998), *Alcaligenes*

eutrophus JMP134 (gene *tfdF*; Kasberg *et al.*, 1995) and the *clcE* gene from *Pseudomonas* sp. strain B13 (Kasberg *et al.*, 1997) and it could be involved in the biodegradation pathways of a variety of organic chemicals (Seibert *et al.*, 2004).

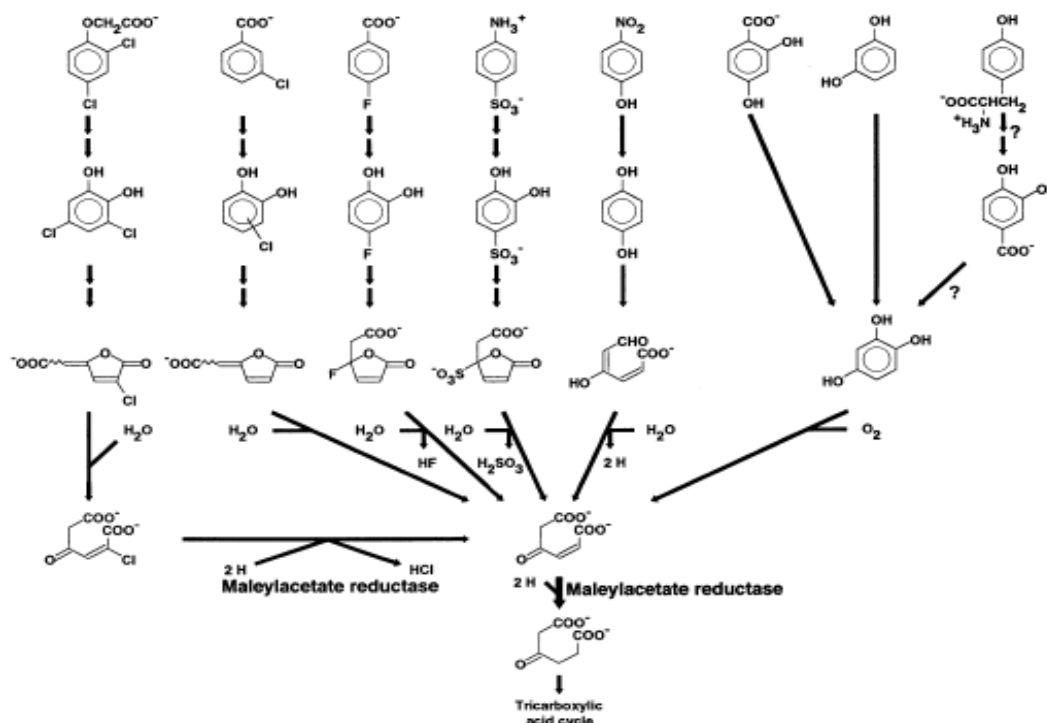


Figure 1.7: Involvement of maleylacetate reductase in bacterial catabolic pathways of aromatic compound degradation. Source: Seibert *et al.*, 1993.

1.10. Research area

1.10.1. River Dene

The **River Dene** (Figure 1.8 b) is a small river in Warwickshire, UK. It is a tributary of the Avon (Figure 1.8 a), which it joins at Charlecote Park. The headwaters of the River Dene rise on the western slopes of the Burton Dassett Hills and flow westward towards Kineton. Five miles downstream of Kineton, the river turns abruptly north, flowing through the villages of Walton and Wellesbourne before joining the Avon. To the west of Kineton, the river was bridged in numerous places, by the Stratford-upon-Avon and Midland Junction Railway. The remains of four sets of sluice-gates, possibly used for the washing of sheep, can be seen at Kineton (Wikipedia, 2012 b).

In 2009 all River Dene stretches had very high (5) phosphate levels (**Table 1.7**). The nitrates grade ranged from moderately low (3) to high (5). Biology grade was found to be fairly good (c) in upstream part of River Dene. The biology improved downstream to the very good grade a, which is similar to that expected for an unpolluted river. Around Wellesbourne, River Dene had fairly good to good biology grade (c/b). The chemistry classification of River Dene ranged from very good (a) to good (b) from upstream to downstream stretches of that river (Environment Agency, 2012 a).

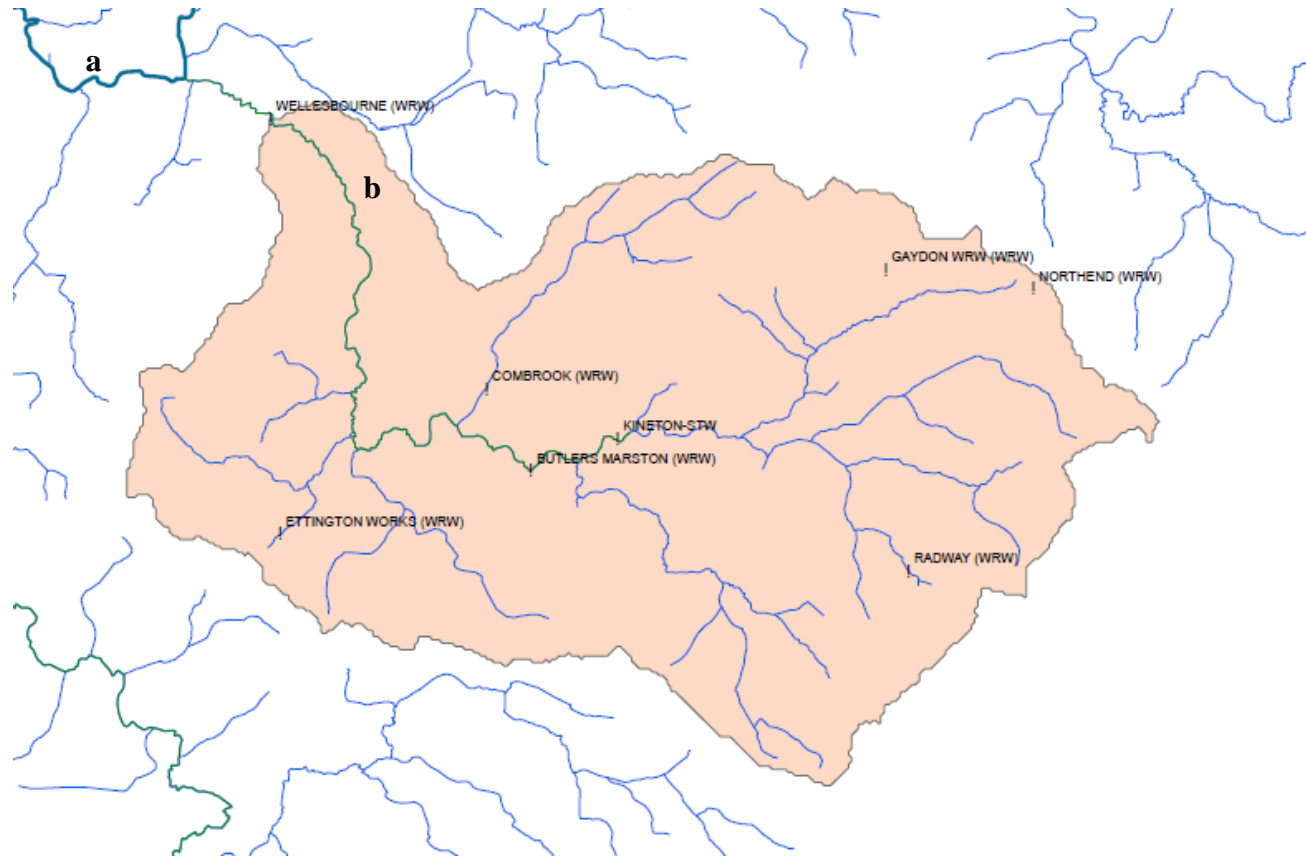


Figure 1.8: River Dene catchment with local Wastewater Treatment Plants. Where: **a**- River Avon (blue), **b**- River Dene (green). Source: Williams, R., Centre for Ecology and Hydrology, UK.

Table 1.7: Water quality data for River Dene. Source: Environment Agency, 2012 b.

River stretch	Chemistry		Chemistry grade	Biology		Biology grade	Nitrates/grade	Phosphates/grade
	Average ammonia (mg NL)	Average dissolved oxygen (% saturation)		NTAXA	ASPT			
Wellesbourne to R. Avon	0.058	88.5	A/B	73	88	b/c	5	5
Thornton to Wellesbourne Mill	0.06	89.72	A/B	29	5.62	a/a	4	5
Below Kineton to Trib. From Thornton	0.046	88.44	A/B	22	5.5	a/a	4	5
Near Kineton to Below Kineton	0.208	93.97	B/B	26	5.08	b/b	5	5
At Northend to Near Kineton	0.34	97.43	A/A	18	4.72	c/c	3	5

Where: NTAXA- number of taxa; ASPT- average score per taxon; **Chemistry grade (Ammonia/Dissolved Oxygen):** A-good, B- fairly good; **Biology grade:** a- very good, b- good, c- fairly good; **Nitrates/grade** and **Phosphates/grade:** 3- moderately low, 4- medium, 5- high.

1.10.2. Wastewater Treatment Plants (WWTPs)

There are eight WWTPs located within River Dene catchment (**Figure 1.9**) with a variety of treatment strategies and population sizes from which the wastewater is received (**Table 1.8**). Wellesbourne WWTP (**Table 1.8**) is the largest WWTP discharging treated effluent directly to River Dene. Population equivalent (receiving population) to this WWTP was 6,924 people in 2010. This WWTP has got a secondary treatment using Activated Sludge. Tertiary treatment stage also may use

rapid-gravity sand filters, moving bed filters, pressure filters, and nutrient control using physico-chemical and biological methods, disinfection, COD and colour removal (Johnson *et al.*, 2007).

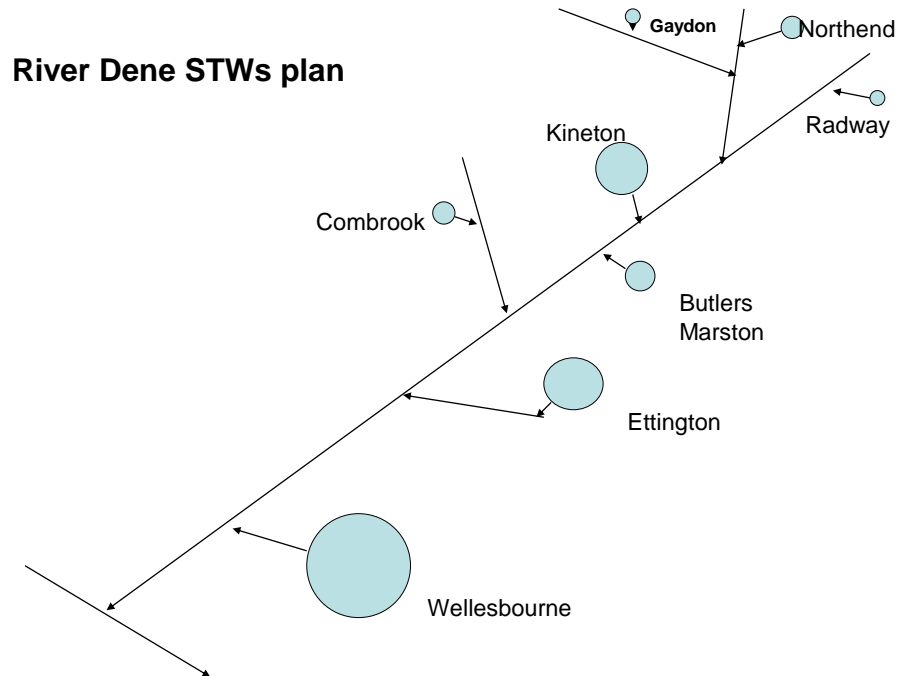


Figure 1.9: River Dene catchment with Wastewater Treatment Plants.
Source: Severn Trent Water, UK, where: STWs – Sewage Treatment Works.

1.10.3. Flood events

The 20th July 2007, saw a deluge of heavy and persistent rainfall over central and southern England. This caused extensive flooding over the River Dene and River Avon catchments and severe traffic disruption. In July 155 mm of rainfall was recorded at Wellesbourne which is over three times the long term average for the area. As a result of this heavy rainfall, river levels rose at extreme rates to some of the highest ever recorded. In Wellesbourne the River Dene reached a peak level of 43.5 m. This is higher than the flood of April 1998 when the river peaked at a level of 43.3 m. The July flood on the River Dene at Wellesbourne has been assessed as having a 0.6 per cent probability of occurring in any year (Environment Agency, 2012 b).

Table 1.8: Wastewater Treatment Plants within River Dene catchment.

Treated effluents are directly discharged to River Dene (Butlers Marston, Kineton and Wellesbourne WWTPs) or to the tributary of River Dene (Combrook, Ettington, Northend and Radway WWTPs) as well as to Gaydon brook (Gaydon WWTP). Source: Severn Trent Water, UK.

Works	Type of treatment	Treatment category	Discharge to	Population equivalent*
Radway	trickling filter + reed bed	TB1**	Tributary of R. Dene	268
Northend	RBC + reed bed	TB1**	Tributary of R. Dene	391
Gaydon	RBC + reed bed	TB1**	Gaydon brook	395
Kineton	trickling filter	TB1**	R. Dene	2,536
Butlers Marston	trickling filter + reed bed	BIO**	R. Dene	556
Combrook	trickling filter + reed bed	TB1**	Tributary of R. Dene	78
Ettington	RBC + reed bed	TB1**	Tributary of R. Dene	894
Wellesbourne	nitrifying ASP	TA2**	R. Dene	6,924

* receiving population in 2010 - population connected to WWTP, ** specific treatment category is unknown, but general information on each treatment type is provided where: **RBC**- rotating biological contactor; **BIO**- biological; **TB1**- works with a secondary stage biological process whose treatment methods also include prolonged settlement in conventional lagoons or raft lagoons, irrigation over grassland, constructed wetlands, root zone treatment (where used as a tertiary stage), drum filters, microstrainers, slow sand filters, tertiary nitrifying filters, wedge wire clarifiers or Clariflow installed in humus tanks, where used as a tertiary treatment stage; **TA2**- Works with a secondary activated sludge process whose treatment methods also include prolonged settlement in conventional lagoons or raft lagoons, irrigation over grassland, constructed wetlands, root zone treatment (where used as a tertiary stage), drum filters, microstrainers, slow sand filters, tertiary nitrifying filters, wedge wire clarifiers or Clariflow installed in humus tanks, where used as a tertiary treatment stage (Johnson *et al.*, 2007).

1.11. Aims and objectives of the project

The major aims of the current PhD project were to improve understanding of pollutant biodegradation mechanisms occurring in rivers, and to identify bacterial populations involved in these processes in order to identify factors which could increase the realism and reproducibility of chemical biodegradation tests.

The specific objectives were:

1. Development of a functional marker approach to study the diversity of *para*-nitrophenol (PNP) degrading bacteria present in environmental samples.
2. Determine the effect of light on the chemical biodegradation
3. Determine the effect of inoculum material, sampling site location and sampling time on the chemical biodegradation.
4. Development of novel test systems utilising biofilms generated in the field to investigate chemical biodegradation
5. Determine whether chemical biodegradation kinetics can be related to dynamics of specific degraders within the community.
6. Determine the effect of chemical concentration on the rate of biodegradation and identify the threshold chemical concentration.

Chapter 2

The biodegradation of *para*-nitrophenol under light and dark conditions: a functional gene approach.

2.1. Introduction

Development of the Chemical Industry over the past few decades has resulted in production and application of new chemical compounds which now can be found in a range of everyday use products ranging from pharmaceuticals (Vieno *et al.*, 2006) to personal care (Ramirez *et al.*, 2009) and household products (Fenet *et al.*, 2003). Due to the nature of their application, such chemicals enter the environment primarily via wastewater. In order to determine the persistence and potential toxic effects of chemicals in the environment, standardized biodegradation tests have been established by the Organisation for Economic Cooperation and Development (OECD).

These tests are usually conducted under environmentally unrealistic conditions. For instance, light is excluded from tests or diffuse light is recommended in OECD biodegradation tests to avoid any potential test failure caused by overgrowth of algae. Light is a major concern in biodegradation studies and particularly in tests that are using respirometric methods to assess the biodegradation of chemicals, where interference from increased dissolved oxygen may occur due to the photosynthesis conducted by algae. Dissolved oxygen also affects aerobic chemical biodegradation, since oxygen serves as an electron acceptor in the biodegradation processes (Xu *et al.*, 2005). Algae developing under light conditions may also compete with bacteria for the energy source, and may impact biodegradation kinetics of test chemicals. Light is also responsible for direct and indirect photolysis of chemicals. Direct photolysis is light-dependent degradation of chemicals while indirect photolysis is caused by light-induced growth of phototrophs which are capable of chemical biodegradation. Light can induce radicals which are involved in oxidation of nitrophenols (Kiwi *et al.*, 1994). Additionally, light in combination with sensitizers

can stimulate the photodegradation of chlorophenols (Bandara *et al.*, 2001). Light is responsible for photochemical reactions which occur near the water surface e.g. in river water (Klöpffer, 1992) where chemicals may undergo photo-oxidative degradation (Datta *et al.*, 2004) and photocatalytic degradation (Li *et al.*, 2005). Interestingly, light conditions may also favour the biodegradation of chemicals by phototrophs i.e. algae may influence photolysis rates of chemicals in water. This is due to the light induced algal photosynthesis which results in enhanced algal growth. Zepp and Schlotzhauer (1983) reported that from the group of 22 compounds that they studied accelerated light-dependent degradation of certain organic chemicals was observed in the presence of green and cyanobacteria at concentration of 1-10 mg of chlorophyll *a*/L.

Phototrophic microorganisms have been reported as capable of chemical biodegradation under light conditions by Roldán *et al.* (1998) who demonstrated the degradation of PNP by the bacterium *Rhodobacter capsulatus* which was incubated in the presence of light. Two species of microalgae, *Chlorella vulgaris* var. *vulgaris* f. *minuscule* and *Coenochloris pyrenoidosa* were isolated by Lima *et al.* (2003) from microalgal consortium which degraded PNP. Both were capable of PNP degradation when cultured separately. Hirooka *et al.* (2006) reported that from the mixture of photoautotrophic microorganisms two microorganisms *Chlamydomonas reinhardtii* and *Anabaena cylindrica* had the highest ability to remove 2,4-dinitrophenol (2,4-DNP) and 2-amino-4-nitrophenol (2-ANP) in liquid medium under light conditions. Algae are also capable of biodegradation of aromatic chemicals in the mixed algal-bacterial culture systems (Borde *et al.*, 2003). These results prove that algae can also play a key role in the biodegradation of chemicals present in surface water, and therefore, should not be excluded in the biodegradation tests.

Absence of light is not the only factor which makes OECD biodegradation tests unrealistic. The amount of chemical applied in biodegradation tests may cause difficulties with data interpretation and extrapolation of results to environmental compartments. In wastewater treatment or in the environment many chemicals are present at ng/L to µg/L levels whereas in the OECD tests the concentrations are at the level of mg/L (Ahtiainen *et al.*, 2003). The reason biodegradation tests have developed in this manner is that dosing at realistic concentrations requires labelled compounds and specific analysis, and therefore, more convenient approach was

developed for environmental risk assessment of thousands of compounds. Kinetics of biodegradation of chemicals and chemical bioavailability depend on chemical concentration. Biodegradation of a chemical at higher concentrations is usually a growth-linked process (Rhee *et al.*, 2002; Abuhamed *et al.*, 2004) whereas at low concentrations chemicals do not serve as primary substrates in the environment where a variety of compounds are present (Ramakrishnan *et al.*, 2011). Therefore, cometabolism may exist at lower concentrations of the primary compound which results in lower chemical biodegradation rates (Wang *et al.*, 1984; Novic and Alexander, 1985). In addition, very low chemical concentrations affect the rate of entry of substrate into the cell (Battersby, 1990), and since many microbial transporters and catabolic enzymes are regulated and are synthesized in response to the presence of a certain concentration of their substrate (Harms and Bosma, 1997) the induction of enzymes which are involved in the biodegradation pathways of test compounds might require higher concentration of chemical (Hanne *et al.*, 1993).

Application of high levels of test compound can be useful in identifying populations that biodegrade the compound and can also be used to assess differences in biodegradation potential of inocula from different environmental compartments (Johnson *et al.*, 2004). However, high chemical concentration cannot be used for the prediction of chemical biodegradation under environmental conditions, since, for reasons explained above, biodegradation may not reflect the process under typical environmental conditions. Moreover, false negative test results may be generated with high concentrations if these are toxic to the microbial inoculum (Comber and Holt, 2010). Hence, realistic concentrations of chemical should be introduced into biodegradation systems for better understanding of processes occurring under environmentally relevant conditions.

The most direct approach for identifying chemical-degrading consortia is by analysis of functional genes that encode key enzymes in degradation of such chemicals. Some authors have already applied the functional gene approach to their biodegradation studies. Piskonen *et al.* (2008) used naphthalene dioxygenase and toluene dioxygenase genes for monitoring efficiency of toluene and naphthalene biodegradation in soil and Zhou *et al.* (2006) investigated diversity of bacteria degrading polycyclic aromatic hydrocarbons in mangrove sediments, using dioxygenases as functional markers. In the current project, *para*-nitrophenol (PNP)

was chosen as a model compound and procedures were developed to use genes involved in PNP biodegradation pathways as functional markers to study PNP-degrading bacterial populations in biodegradation experiments inoculated with environmental communities.

In addition, application of emerging high throughput sequencing methods in combination with the functional gene analysis was used in this project. High throughput sequencing is a powerful technique which enables fast generation of a large amount of sequencing data from samples. These methods have been reported as useful tools to study in depth populations involved in biodegradation processes (Iwai *et al.*, 2010; Warnecke and Hess, 2009), tracking shifts in distribution and structure of microbial communities caused by a variety of environmental factors (Acosta-Martínez *et al.*, 2008, Mackelprang *et al.*, 2011), linking the community structure and function (Fuhrman, 2009) and detection and identification of uncultivable degraders (Eyers *et al.*, 2004; Chen and Murrell, 2010).

2.2. Objectives

The major objective of this experimental work was to determine the effect of two variables, light and PNP concentration on the biodegradation of PNP. The following hypotheses were tested (1) light does not alter the rate of the biodegradation of *para*-nitrophenol (PNP), (2) the biodegradation of PNP is not affected by PNP concentration (3) the composition of river water bacterial community is not affected by light and/or PNP concentration. Functional marker approach was developed for analysis of *pnpA* and *mar*, and was applied to identify PNP-degraders and to establish key organisms involved in catabolism of PNP in river water.

2.3. Materials and methods

2.3.1. Sample collection

In March 2010, samples were obtained from the River Dene 530 m downstream of the Wellesbourne Wastewater Treatment Plant (WWTP) effluent discharge point (52°11'58.54''N and 1°36'44.94''W). The river water collected was filtered through a 38 µm sieve in order to remove particles and large protozoa and then was used as inoculum in PNP biodegradation experiments. River water for the controls was sterilized in three autoclaving cycles (121 °C, for 15 minutes, 1.1 atm). Biodegradation tests were carried out according to guideline OECD 309.

2.3.2. Experimental set up

Sterile Duran Schott amber and clear glass bottles (500 mL) (Fisher Scientific, UK) were used to simulate dark and light conditions, respectively. Bottles were filled with river water as an inoculum (300 mL). Treatments and controls were prepared in triplicate. PNP, (**Table 2.1**) was used to prepare an aqueous solution containing 1200 mg/L and 12 mg/L of PNP which was used for spiking water to the initial PNP concentrations of 2 mg/L (high concentration) and 20 µg/L (low concentration), respectively. The ratio between the total capacity of the bottle (500 mL) and the volume of river water (300 mL) matched that of Toräng and Nyholm, (2005). A positive control was set up consisting of sterile river water with PNP inoculated with induced PNP-degrading bacterial strains *Pseudomonas putida* SoT01 and *Arthrobacter* sp. JS443 (**Table 2.2**), kindly provided by Dr Liz Shaw (University of Reading, Department of Soil Science, UK), grown on LB medium with PNP (56 mg/L) for 48 hours at 25°C. Negative controls consisted of sterile river water with PNP. The experiment (**Figure 2.1**) was run in a controlled environment room with an 18 hour light (white light source with intensity of 7450 lux) and six hour dark cycle. The incubation temperature was maintained at 20±2 °C, and bottles were incubated under constant motion using the rotary shaker (MK V Shaker, L.A. Engineering Co., UK) at 50 rpm. River water with high and low PNP concentration was incubated for eight days in the dark (until biodegradation of PNP) and 36 days

under light conditions, due to lack of PNP biodegradation after the 28 day incubation. River water pH was measured before and after dark and light incubation using Hanna Instruments 222 Calibration Check pH meter (MBH Engineering Systems, USA)



Figure 2.1: River water incubated under light and dark conditions with PNP.

Table 2.1: Chemical compounds used in this study. Synthesised by Sigma-Aldrich, UK.

Chemical	Reagent grade (%)	Retention time (minutes)
<i>Para</i> -nitrophenol	99	7.8
<i>Para</i> -benzoquinone	98	5.3
Hydroquinone	97	4.9
4-nitrocatechol	97	4.0

Table 2.2: Strains used in this study.

Strain	Source	Gram reaction	Growth medium	Application
<i>Arthrobacter</i> sp. JS443	University of Reading, Department of Soil Science, UK	+	LB + PNP	Positive control in biodegradation experiments Primer optimisation
<i>Arthrobacter</i> <i>chlorophenolicus</i> A6	DSMZ 12829 Westerberg <i>et al.</i> , 2000	+	LB + PNP	Primer optimisation
<i>Pseudomonas</i> sp. SoT02	University of Reading, Department of Soil Science, UK	–	LB + PNP	Primer optimisation
<i>Hoeflea</i> <i>phototrophica</i> DFL- 43	DSMZ 17068 Biebl <i>et al.</i> , 2006	+	LB + PNP	Primer optimisation
<i>Rhodococcus</i> <i>opacus</i> B4	DSMZ 44313 Klatte <i>et al.</i> , 1994	+	LB + PNP	Primer optimisation
<i>Burkholderia</i> <i>xenovorans</i> LB400	DSMZ 12829 Goris <i>et al.</i> , 2004	–	LB + PNP	Primer optimisation

DSMZ- German Collection of Microorganisms and Cell Cultures

2.3.3. Measurement of PNP biodegradation

Daily, 1 mL aliquots of water were removed from each bottle and PNP was quantified by HPLC. The analysis was carried out using a Hewlett Packard 1100 HPLC system with a LiChrosphere (5 μ m) C-18 column (MerckMillipore, UK). The mobile phase was a mixture of water: methanol (HPLC grade, Fisher Scientific, UK) with a ratio of 40:60 and the flow rate was 0.50 mL/minute. Analysis was performed with UV detection at 254 nm, with column pressure of 115 bar and column temperature at 25 °C. The volume of the injected sample was 20 μ L and the run time was 13 minutes. Standard solutions were prepared containing PNP and the metabolic intermediates *para*-benzoquinone (pBQ), hydroquinone (HQ), and 4-nitrocatechol (4-NC) (**Table 2.1**) HPLC calibration was carried out using concentrations of 2 mg/L to 2 μ g/L for PNP and 2 mg/L for all other compounds. pBQ, HQ, 4-NC, were detected by UV spectrometry at 254 nm and the retention times were determined (**Table 2.1**). The detection limit was defined as the lowest concentration of PNP at which PNP presence/absence can be distinguished on the chromatogram and it was found at PNP concentration of 2 μ g/L. However, interference from unknown compounds in environmental samples (sample background interference) caused difficulties with PNP measurements below 20 μ g/L. No PNP metabolites were detected in any of the experimental samples.

2.3.4. Flow cytometry

The total number of cells, numbers of algae and bacteria were assessed using flow cytometry. In order to obtain concentrated biomass from water samples, 100 mL aliquots of river water were taken before incubation (T_0) and after complete PNP biodegradation. Millipore GVWP04700, (hydrophilic Durapore) polyvinylidene fluoride membrane filters (0.22 μ m mesh, 47 mm diameter), attached to a vacuum source were used. Cell fixation with 1% w/v paraformaldehyde (PFA) (Sigma-Aldrich, UK) was performed prior to measurement. For the flow cytometry analysis 1 mL of sterile water and river water samples were stained with 200 μ L solution which consisted of 100 μ L of Syber GreenX10 (Invitrogen, UK), 10 μ L of

fluorescence beads (0.5 μm) (Becton Dickinson, UK) and 1 mL of trisodium citrateX10 (Fisher, UK). After 30 minutes of incubation at room temperature sterile water with beads was used for instrument calibration followed by analysis of stained river water samples which were run for 2 minutes. A distinct clusters of bacterial and algal cells were determined in the cytogram based on the differences in their cell size. The number of cells have been counted within each cluster using BD FACScan Machine, using 488 nm laser (Becton Dickinson, USA).

2.3.5. Determination of total viable count (colony forming units)

Plating for bacterial colonies was used as a culture dependent approach to determine the number of bacteria (inoculum size) present in river water and for isolation of PNP-degrading bacteria after PNP biodegradation.

2.3.5.1. Inoculum size

Bacterial cell density in river water was determined using freshly collected samples of river water. Aliquots of a 10-fold serial dilution of river water in sterile PBS (phosphate buffered saline, pH=7.0) were spread onto duplicate plates of R2A agar (OXOID, UK). After incubation at 25 °C for 10 days bacterial colonies were counted.

2.3.5.2. Isolation of PNP-degrading bacteria

For isolation of PNP-degrading bacteria, aliquots of river water were taken from amber and light glass bottles after PNP biodegradation and at the end of incubation from river water treatments which did not degrade PNP. Serial dilutions were spread onto plates with solidified mineral medium (NH_4Cl , 0.4 g/L; $\text{MgSO}_4 \times 7\text{H}_2\text{O}$, 0.02 g/L; trace elements solution, 2 mL/L; KH_2PO_4 , 0.3 g/L; Na_2HPO_4 , 1.24 g/L) and PNP (56 mg/L) as a sole source of carbon. Every 24 hours of incubation at 25 °C plates were examined and those with clear zones (disappearance of yellow colour

due to PNP biodegradation) were chosen for isolation of PNP degrading bacteria. Colonies with clear zones were selected (representative colonies for each morphological type: size, colour and texture) and were cultured on Luria-Bertani agar (LB agar, Sigma-Aldrich, UK) with PNP (56 mg/L). After 48 hours of incubation at 25 °C, pure cultures of the isolates which degraded PNP were obtained. Single colonies were selected from subsequent pure cultures for identification.

2.3.6. Colony PCR

Single colonies from pure cultures of bacterial isolates were re-suspended in 100 µL of sterile water. Colony PCR was performed with universal primers for bacterial 16S rRNA gene amplification (**Table 2.3**). The PCR reaction cocktail consisted of 43 µL of MegaMix (Microzone Limited, UK), 1 µL of each primer at a concentration of 5 µM, and 5 µL of colony suspension. The PCR program used for 16S rRNA gene amplification was as follows; 95 °C for 3 minutes, 30 cycles of 95 °C for 30 seconds, 55 °C for 1 minute, 72 °C for 1 minute and 10 minutes at 72 °C. Products were purified using a QIAquick PCR purification kit (Qiagen, UK) and DNA concentration was measured by NanoDrop ND-1000 Spectrophotometer (Thermo Scientific Fisher, UK). A single sequencing reaction had a final volume of 10 µL and consisted of: 2 µL of BigDye (V3.1. Applied Biosystems, UK), 2 µL of 5X sequencing buffer (Applied Biosystems, UK), 1 µL of 63F or 1087R primer (Hilton *et al.*, 2013) and 5 µL of PCR product (20 ng/µL). The following PCR program for sequencing was used; 96 °C for 1 minute, 25 cycles of 96 °C for 10 seconds, 50 °C for 5 seconds, 60 °C for 4 minutes. Sequences obtained after sequencing reactions with forward and reverse primers were used to create contigs with DNASTAR Lasergene 8 (SeqEdit). The sequence data obtained in the manner described above were analysed using 16S rRNA sequence data available online in public databases of National Centre for Biotechnology Information (NCBI) using Basic Local Alignment Search Tool (BLAST).

Table 2.3: Primers used in this study. Synthesised by Invitrogen, UK.

Primer name	Sequence (5'→ 3')	Specificity
63F-NED	CAGGCCTAACACATGCAAGTC	Bacteria 16S
1087R-VIC	CTCGTTGCGGGACTTACCCC	Bacteria 16S
M13F	GTTTTCCCAGTCACGAC	pDrive vector
M13R	CAGGAAACAGCTATGAC	pDrive vector
pnp349F	AACGGYGARCTGATCGCGAAGAT	<i>pnpA</i>
pnp635R	GTGCTGCGRGCRCCTCGGT	<i>pnpA</i>
npd276F	CTCCAGGGMGGYATCTTCAC	<i>npdA2</i>
npd865R	TCTCRAKGTGBACCCAGTCGAA	<i>npdA2</i>
marB349F	CCNACSACSTAYGCVGGNTC	<i>mar</i>
marA715R	RTGRCAVAGYTTGTGRTG	<i>mar</i>

Where: F- forward primer, R- reverse primer, pnp – primers targeting *pnpA* gene, npd- primers targeting *npdA2* gene; mar – primers targeting *mar* gene; M13- vector targeted PCR primers, VIC and NED – fluorophore labels; numbering according to nucleotide sequences.

2.3.7. ¹⁴C PNP biodegradation assay with bacterial isolates

In order to test the potential of isolated bacterial strains for PNP biodegradation a ¹⁴C PNP assay was used. The following three bacterial isolates which showed PNP biodegradation on mineral medium with PNP were chosen: *Pseudomonas syringae* (AKHD2), *Pseudomonas fluorescens* (AKHD1) and *Bacillus pumilus* (AKLD2).

2.3.7.1. Experimental set up

Sterile amber Duran Schott bottles (200 mL) (Fisher Scientific, UK) were filled with sterile mineral medium (20 mL). Triplicates of bottles with mineral medium inoculated with each tested isolate were prepared. For the positive control, mineral medium was inoculated with the PNP-degrading bacterial strain *Arthrobacter* sp. JS443 (**Table 2.2**). The negative control consisted of sterile mineral medium with ^{14}C PNP. Single positive and negative controls were prepared. PNP was used for preparing aqueous solutions containing 1200 mg/L and 120 $\mu\text{g/L}$ of substrate which was used for spiking water to the initial concentration of PNP at 2000 $\mu\text{g/L}$ (high) and 0.2 $\mu\text{g/L}$ (low), respectively. Additionally, a 500 μL stock solution of ^{14}C PNP with specific activity of 55 mCi/mmol (American Radiolabeled Chemicals Inc., USA) was used to spike the mineral medium to the initial radiation of 0.2 kBq/mL. The amount of ^{14}C PNP added was 2.04 MBq/mol PNP per bottle which gave 0.6 and 99.8 % of total PNP present in treatments with total PNP concentration at 2014 $\mu\text{g/L}$ and 14 $\mu\text{g/L}$, respectively. The bottles were closed using screw caps with attached $^{14}\text{CO}_2$ traps. The traps consisted of scintillation vial containing 1 mL of 1M NaOH (Fisher Scientific, UK). Incubation conditions were as described in section 2.3.2.

2.3.7.2. Measurement of PNP and ^{14}C PNP biodegradation

The biodegradation of PNP was monitored as previously described in section 2.3.3. The biodegradation of parent ^{14}C PNP was monitored using scintillation counter measurements. Daily, 1 mL of medium was removed from bottles and placed in a scintillation vial (National Diagnostics, UK) followed by the addition of 10 mL of scintillation liquid Ecoscint A (National Diagnostics, UK) and measurement was made using a scintillation counter Packard Tri-Carb 2900-TR (PerkinElmer Inc., USA) using protocol for ^{14}C counts. Also, evolution of $^{14}\text{CO}_2$ from ^{14}C PNP biodegradation was monitored. Daily, traps from the bottles, containing 1 mL of 1M NaOH, were removed from each bottle and replaced with fresh ones. 10 mL of Scintillation liquid Ecoscint A was added followed by overnight incubation at room

temperature. $^{14}\text{CO}_2$ was measured on a scintillation counter using the method described above.

2.3.8. Effect of light and pH on PNP biodegradation

In order to determine the direct effect of light and pH on the biodegradation of PNP a biodegradation test with the PNP-degrading isolate *Pseudomonas syringae* AKHD2 was performed in liquid mineral medium adjusted to low (7.5) and high (9.8) pH using 1M HCl (Fisher Scientific, UK) and 1M NaOH (Fisher Scientific, UK). Treatments were prepared in triplicate and consisted of sterile Duran Schott amber and light glass bottles (100 mL) (Fisher Scientific, UK) filled with sterile mineral medium (60 mL) inoculated with induced PNP-degrading bacterial isolate, *P. syringae* AKHD2. Negative controls consisted of mineral medium with adjusted pH. PNP was used for preparing aqueous solution containing 240 mg/L substrate which was used for spiking liquid medium to the initial concentration of PNP at 2 mg/L. Incubation conditions and measurements were performed as described in sections 2.3.2. and 2.3.3., respectively.

2.3.9. Terminal Restriction Fragment Length Polymorphism (TRFLP)

Aliquots of freshly collected river water and residual water from bottles after incubation under dark and light with PNP were filtered using Millipore GVWP04700, (hydrophilic Durapore) polyvinylidene fluoride membrane filters (0.22 μm mesh, 47 mm diameter), attached to a vacuum source. DNA was extracted using the PowerWater[®] DNA extraction kit available from Mo-Bio Laboratories (Cambio, UK), using the manufacturer's recommended protocol. Extracted DNA was used as a template in PCR where the 16S rRNA gene was amplified for Terminal Restriction Fragment Length Polymorphism analysis using universal bacterial primers: 63F-NED (Marchesi *et al.*, 1998) labelled and 1087R-VIC (Hauben *et al.*, 1997) labelled (**Table 2.3**). The PCR reaction cocktail consisted of 47 μL of MegaMix, 1 μL of each primer at a concentration of 5 μM , and 1 μL of template DNA at a concentration of 10 ng/ μL . The final reaction volume was 50 μL . The PCR program used for 16S rRNA gene amplification was as follows; 95 °C for 3

minutes, followed by 30 cycles of 95 °C for 30 seconds, 55 °C for 1 minute, 72 °C for 1 minute and 10 minutes at 72 °C. The PCR reaction was carried out in duplicate for each sample and the resulting products were pooled and purified using a QIAquick PCR purification kit (Qiagen, UK) using the manufacturer's recommended protocol to remove unincorporated nucleotides and primers. The DNA concentration was measured at 260 nm using a NanoDrop ND-1000 Spectrophotometer (Thermo Scientific Fisher, UK) prior to restriction digestion. PCR products (400 ng/μL of DNA) were digested for 4 hours at 37 °C in a 20 μL reaction volume followed by the enzyme inactivation at 95 °C for 15 minutes. Restriction enzymes used were: *HhaI* (2U) and *MspI* (5U) (New England BioLabs Ltd., UK) with restriction sites 5' – GCG/C and 5' – C/CGG, respectively. After restriction digest, 0.015 μL of molecular standard LIZ 1200 (Applied Biosystems, UK) was added to each sample and 1 μL of sample was run on a Genetic Analyser ABI PRISM 3130xl (Applied Biosystems, UK) equipped with 36 cm capillary array. Restriction fragments were size-separated and compared with internal lane molecular size standard (LIZ 1200). Fragment separation data were analysed using GeneMarker® (AFLP Genotyping Software version 1.6).

2.3.10. Primer design for *pnpA*, *npdA2* and *mar*

Sequences for PNP functional genes analysed by Zhang *et al.*, (2009) from Gram-negative and Gram-positive bacteria (**Figure 2.2**) were downloaded from the NCBI and aligned using ARB, a software environment for sequence data (Ludwig *et al.*, 2004). Searches were carried out using blastp and blastn available from the BLAST (Altschul *et al.*, 1990) with representative sequences taken from the relevant database on NCBI, in order to identify additional sequences of *pnpA* and *npdA2* in the database. Search terms consisted of names of enzymes encoded by gene *pnpA* and *npdA2*, PNP 4-monooxygenase and 2-monooxygenase. Additional sequences were collected using BLAST search for accession numbers for enzymes encoded by *pnpA* and *npdA2* gene reported by Zhang *et al.*, (2009). Sequences of these genes were then uploaded into ARB software version 5.3-private-8210 (Ludwig *et al.*, 2004), translated into amino acid sequences and used to generate an alignment using ClustalW option for protein alignment (slow alignment) in ARB software (Ludwig *et*

al., 2004). Nucleic acid sequences were then re-aligned according to aligned amino acid sequences. The phylogenetic neighbour-joining tree with *pnpA* reference sequences (**Appendix Figure 0.2**) was created using build neighbour-joining tree option available in ARB (Ludwig *et al.*, 2004). Point Accepted Mutation (PAM) correction was used to score sequence alignments and to create the distance matrix. The tree was build based on the conserved amino acid sequence (position 30-398, 251 valid columns) using *pnpA* sequence from *Pseudomonas* sp. WBC-3 as a filter. *Penicillium chrysogenum* Wisconsin was selected as outgroup, since on the blastp search with *pnpA* sequence from *Pseudomonas* sp. WBC-3 there was a clear separation in terms of identity and similarity values that distinguish fungal *pnpA* sequences from the *Pseudomonas* sequences. Sequence alignment for *npdA2* gene and a new neighbour-joining tree were created as described above. The tree (**Appendix Figure 0.3**) was build based on the conserved amino acid sequence (position 42-552, 419 valid columns) and using *npdA2* sequence from *Arthrobacter chlorophenolicus* A6 as a filter. *Burkholderia cepacia* was selected as outgroup. Bootstrap analysis (100 replicates) was performed for *pnpA* and *npdA2* tree using ARB software (Ludwig *et al.*, 2004).

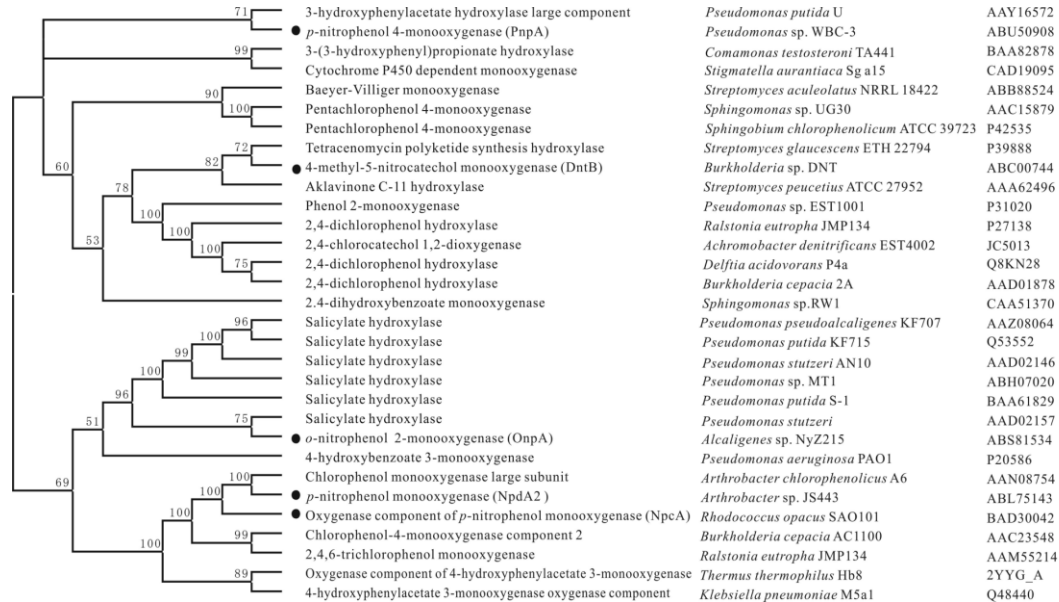


Figure 2.2: Phylogenetic relationship of the nitroarene monooxygenases and their homologues.

Five nitroarene monooxygenases (filled circles), as well as the homologues, were aligned using the Cluster W program (version 1.8) (Thompson *et al.*, 1994) with default settings. A distance neighbour-joining tree was then created using the Mega (version 3.1) package (Kumar *et al.*, 2004), and the bootstrap confidence limits (expressed as percentages) are indicated at the nodes. Enzymes accession numbers are presented on the right side. Source: Zhang *et al.*, 2009.

Conserved stretches of aligned amino acid sequences were used to design manually primers for *pnpA* and *npdA2* gene based on database in ARB (Ludwig *et al.*, 2004). In total 25 degenerate primers were designed: 10 forward and six reverse for amplification of the *pnpA* gene fragment (~ 267 bp) (**Appendix Table 0.1**) plus four forward and five reverse primers for amplification of the *npdA2* gene fragment (~ 589 bp) (**Appendix Table 0.2**).

Sequences of maleylacetate reductase were obtained from the Integrated Microbial Genome and Metagenome (IMG) database (IMG, 2010). The search term maleylacetate reductase was used. An alignment with over 50 sequences of that gene from different bacterial species was created in ARB (Ludwig *et al.*, 2004) and a new neighbour-joining tree was created based on aligned amino acid sequences (**Appendix Figure 0.4**) using ARB software (Ludwig *et al.*, 2004) in the same manner as described for *pnpA*. The tree was build based on the conserved amino acid sequence (position 78–562, 331 valid columns) using *mar* sequence from *Rhodococcus opacus* B4 as a filter. *Rhodococcus erythropolis* clade was selected as outgroup. Bootstrap analysis (100 replicates) was performed using ARB software (Ludwig *et al.*, 2004). Conserved stretches of the *mar* amino acid sequence alignment were used to design manually four primers (**Appendix Table 0.3**).

All designed primers for gene *pnpA*, *npdA2* and *mar* were checked for their specificity using primer blast option in NCBI BLAST.

2.3.11. Optimisation of PCR conditions for amplification of PNP biodegradation genes

Optimisation of the newly designed *pnpA*, *npdA2* and *mar* primers was performed in a series of gradient PCR reactions using DNA extracts from control strains of Gram-positive and Gram-negative bacteria (**Table 2.2**) and from the dark bottle isolate *Pseudomonas syringae* AKHD2. The gradient PCR reactions were run using a Gradient Cycler PTC-0200 Peltier Thermal Cycler (BIO-RAD, USA). Different annealing temperature gradients 50-60, 55-65, and 65-70 °C and combinations of different primer pairs were tested. Those sets of primers which resulted in

amplification of a single product (**Figure 2.3**) from a set of representative bacteria that did not yield products with non-target bacteria were chosen for amplification of PNP genes from DNA extracts of isolated bacteria capable of PNP biodegradation and from community DNA extracted from PNP biodegrading experimental samples. Bacterial isolates *Bacillus pumilus* AKLD2 and *Pseudomonas syringae* AKHD2 were used as non-target negative controls for testing designed primers for *pnpA* and *npdA2* gene, respectively. No non-target negative controls were used to test *mar* primers since *mar* gene was detected in all bacterial strains used for primer optimisation.

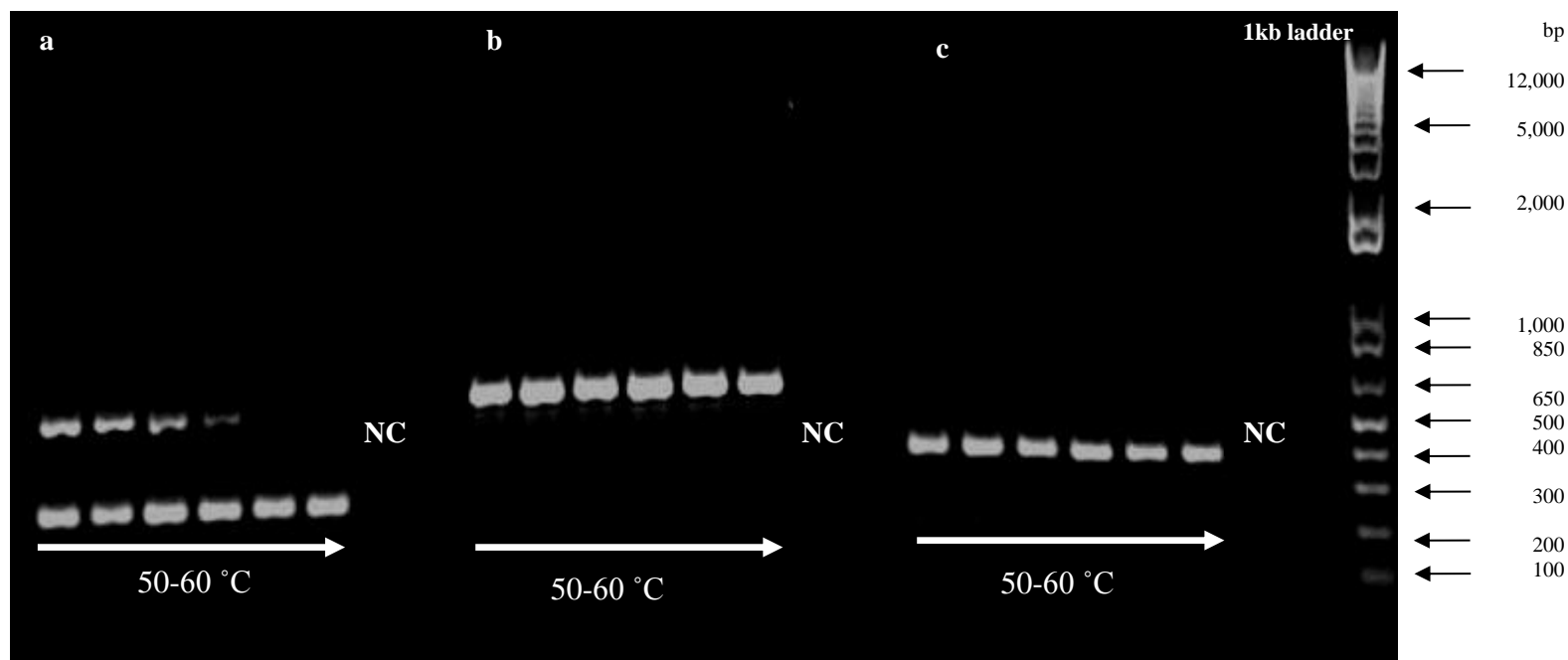


Figure 2.3: Optimisation of PCR reaction conditions from newly designed primers for PNP functional genes involved in PNP biodegradation.

Figure legend: **a-** *pnpA* gene amplified with primer pair pnp349F and pnp635R using DNA template from *Pseudomonas putida* SoT02, **b-** *npdA2* gene amplified with primer pair npd276F and npd865R using DNA template from *Arthrobacter chlorophenolicus* A6, and **c-** *mar* gene amplified with primer pair mar349BF and mar715AR using DNA template from *Rhodococcus opacus* B4, NC – negative control. PCR reactions were performed at gradient annealing temperature (arrows are showing gradient from 50-60 °C). Gel electrophoresis (1% agarose gel (Sigma-Aldrich, UK), run at 90 V, for 50 minutes).

2.3.12. Detection of *pnpA*, *npdA2* and *mar*

Routine amplification of functional genes involved in PNP biodegradation was performed with designed degenerate primers (**Table 2.3**). The PCR reaction had a final volume of 50 μL and consisted of 47 μL of Mega Mix, 1 μL of forward and reverse primer at the concentration of 50 μM , and 1 μL of template DNA at a concentration of 10 ng. The PCR program used for *pnpA* was as follows; 94 °C for 5 minutes, 35 cycles of 94 °C for 1 minute, 72 °C for 1 minute, 72 °C for 1 minute, and 72 °C for 10 minutes. For *mar* gene amplification the following PCR programme was used 94 °C for 5 minutes, 40 cycles of 94 °C for 1 minute, 60 °C for 1 minute, 72 °C for 1 minute, and 72 °C for 10 minutes. All PCR reactions were run on GeneAmp PCR System 9700 (Applied Biosystems, UK).

2.3.13. Clone libraries of *pnpA* and *mar*

PCR products (amplified *pnpA* and *mar* gene) from triplicates of river water before and after dark incubation with 2 mg/L of PNP were pooled together within the same treatment and run on a 1% agarose gel (Agarose, Sigma- Aldrich, UK) at 90 V for 50 minutes. Bands were extracted from the gel using a QIAquick Gel Extraction Kit (Qiagen, UK) according to the protocol provided by the manufacturer. The concentration of PCR products was determined by NanoDrop measurement. Cloning of PCR products was performed with the Qiagen cloning kit (Qiagen, UK) according to the manufacturer's protocol. The final volume of ligation reaction was 10 μL and it consisted of 1 μL of pDrive Cloning Vector (50 ng/ μL), 4 μL of PCR product at a concentration of 40 ng/ μL and 5 μL of 2XLigation Master Mix. The ligation-reaction mixture was mixed and incubated for 30 minutes at 4 °C. Transformation of Qiagen EZ (*E. coli*) Competent Cells was performed. The ligation-reaction mixture (2 μL) was added into each tube with competent cells followed by incubation on ice for 5 minutes, heating the tubes in a 42 °C water bath (SubAqua 12 Grant) for 30 seconds and a final incubation on ice for 2 minutes. Medium SOC (250 μL) was added into each tube and 60 μL of transformation mixture was plated onto LB agar plates containing ampicillin (1 mL/L), X-gal (2 mL/L) and IPTG (200 mM, 250 μL /L) (Sigma-Aldrich, UK). After overnight incubation at 37 °C, LB plates were examined

and for each type of clone, 48 white *E. coli* colonies were picked to perform sequencing of the region containing inserted DNA within plasmid.

Amplification and sequencing of cloned DNA was performed in 96-well plate with an Illustra Templiphi DNA Sequencing Template Amplification Kit (GE Healthcare, UK). Each colony of *E. coli* was transferred to a 5 μ L of Templiphi sample buffer in a 96-well plate. The following PCR program was performed on the GeneAmp PCR System 9700, 95 °C for 3 minutes and cooling to 4 °C. An enzyme mix was prepared by mixing 0.2 μ L enzyme with 5 μ L of reaction buffer and 5 μ L of this mix was added into each well. Incubation was performed using the following PCR program on the GeneAmp PCR System 9700; 30 °C for 18 hours, followed by heating at 65 °C for 10 minutes and cooling to 4 °C.

20 μ L of water was added to each templiphi reaction well prior to sequencing. A single sequencing reaction had a final volume of 10 μ L and consisted of 2 μ L of BigDye, 2 μ L of 5Xsequencing buffer, 1 μ L of 3.2 pmol M13F or M13R primer (**Table 2.3**) and 3 μ L of templiphi reaction. The following temperature program was used for sequencing; 96°C for 1 minute, followed by 25 cycles of 96 °C for 10 seconds, 50 °C for 5 seconds, and 60 °C for 4 minutes.

2.3.14. Phylogenetic analysis of sequences of functional genes

Obtained sequences, 79 for *pnpA* and 62 for *mar* gene, were analysed with Lasergene 10 EditSeq (DNASTAR, USA) and the sequence of the DNA insert was cut out from the sequence of the pDrive vector followed by a blastx search using public databases NCBI BLAST search (Altschul, *et al.*, 1990). DNA sequences which resulted in a positive match with PNP 4-monooxygenase and with maleylacetate reductase were imported to ARB database for *pnpA* and *mar*, respectively, translated into protein sequences and aligned according to the previously established alignments used for designing primers for *pnpA* and *mar* gene, respectively. The phylogenetic neighbour-joining trees were created using amino acid sequence alignment in ARB (Ludwig *et al.*, 2004) in the same manner as described in section 2.3.10. The tree for *pnpA* gene (**Figure 2.10**) was created based on the conserved amino acid sequence (position 89-155, 67 valid columns) using the

pnpA sequence from *Pseudomonas* sp. WBC-3 as a filter. Amino acid sequence alignment was then exported from ARB databases to MEGA 5.1 (Tamura *et al.*, 2011). The evolutionary history was inferred using the neighbour-joining method (Saitou and Nei, 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) are shown next to the branches (Felsenstein, 1985). The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the JTT (Jones Taylor Thornton) matrix-based method (Jones *et al.*, 1992) and are in the units of the number of amino acid substitutions per site. The analysis involved 101 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 61 positions in the final dataset. Evolutionary analyses were conducted in MEGA 5.1 (Tamura *et al.*, 2011).

The phylogenetic neighbour-joining tree of *mar* (**Figure 2.11**) was created using ARB (Ludwig *et al.*, 2004) based on the conserved amino acid sequence (position 133-242, 110 valid columns) and the *mar* sequence from strain *Rhodococcus opacus* B4 as a filter. Representative sequences for OTU 8, 99 and 104 were excluded from the *mar* alignment due to poor sequence quality. Bootstrap values were calculated as described above using MEGA 5.1 (Tamura *et al.*, 2011). The analysis involved 129 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 107 positions in the final dataset.

2.3.15. Quantitative PCR of *pnpA* and *mar* genes

E. coli clones used for *pnpA* and *mar* clone libraries were preserved in 100 µL LB broth with 1 mL/L ampicillin and 50 µL glycerol:water solution (50:50) and stored at -80°C. Certain *E. coli* clones were used as a source of plasmid (pDrive cloning Vector (3.85 kb), Qiagen, UK) inserted with either a fragment of the gene *pnpA* or *mar*. Fresh selective plates (LB agar with ampicillin, X-gal and IPTG added as described for cloning) were streaked with preserved *E. coli* clones. After overnight incubation at 37 °C a single colony was selected to inoculate a starter culture of 3 mL LB medium with ampicillin (1 mL/L). After 8 hours incubation at 37 °C with

vigorous shaking (300 rpm) the culture was diluted 1/500 into selective LB medium as for high-copy plasmids (25 mL LB medium was inoculated with 50 μ L of starter culture). After 14 hours of incubation at 37 °C with vigorous agitation (300 rpm) bacterial cells were harvested by centrifugation at 6,000 \times g for 15 minutes at 4 °C. The cell pellet was frozen and stored at -20 °C for plasmid purification which was performed with Qiagen HiSpeed Plasmid Midi Purification Kit (Qiagen, UK) according to manufacturer's protocol. Plasmid DNA pellet in 50 μ L of TE buffer (pH 8.0) was obtained. Plasmid concentration was determined by NanoDrop measurement. The aliquot with plasmid concentration at 500 ng/ μ L was diluted to about 100 ng/ μ L. Plasmid containing a fragment of the *pnpA* gene was digested with the restriction enzyme *Bam*HI (10 U) (Invitrogen, UK) with a restriction site 5'-G/GATCC, in a 10 μ L reaction which consisted of 0.5 μ L of *Bam*HI, 1 μ L of 10XBuffer, 2 μ L of plasmid (100 ng/ μ L) and water to 10 μ L. The cycle conditions were as follows; digest for 4 hours at 37°C, enzyme inactivation at 95 °C for 15 minutes. Digested plasmid was purified using the PCR purification kit (Qiagen, UK). The concentration of plasmid was confirmed by NanoDrop and appropriate dilutions were performed to obtain a plasmid concentration within the range of 0.002-800 pg/ μ L for the calibration curve in QPCR. Standard plasmid containing fragments of the *mar* gene was obtained in the same way as described for the *pnpA* gene. QPCR reactions were performed in triplicate for negative control, standards and experimental samples. The final volume of reaction was 20 μ L and it consisted of 10 μ L 2XSYBR green (Applied Biosystems, UK), 1 μ L of forward and reverse primers for *pnpA* or *mar* amplification (20 μ M) (**Table 2.3**), 0.2 μ L BSA (40 mg/mL), 1 μ L of template (either standard or sample), and water added to 20 μ L. The QPCR was performed with a Real Time PCR platforms (Applied Biosystems, ABI 7900HT). The cycle conditions were as follows; 95 °C for 10 minutes, followed by 40 cycles of 95 °C for 15 seconds, 60 °C for 1 minute and 95 °C for 15 seconds. For normalization of number of copies of both functional genes, the 16S rRNA gene was amplified with primers included in **Table 2.3**, from DNA extracted from the isolated PNP degrader *Pseudomonas syringae* AKHD2. A known concentration of amplified and purified gene fragment was used for QPCR standard preparation. QPCR for the 16S rRNA gene was performed as described for functional genes. Number of copies of *pnpA*, *mar* and 16S rRNA gene were calculated as detailed by Whelan *et al.* (2003), and were normalized per μ g of extracted DNA. In addition, standard samples

were sequenced by the Genomics Centre at the University of Warwick, School of Life Sciences, Wellesbourne Campus to make sure that the DNA sequence amplified was the same as the one recorded in the clone library sample it was taken from.

2.3.16. 454 sequencing for *pnpA* and *mar* gene

DNA extracts (aliquots of 25 μ L) extracted from triplicate samples of freshly collected river water and from river water which had degraded high PNP concentration in the dark, were submitted to Research and Testing Laboratory (RTL, Texas, U.S.A). Samples were amplified for pyrosequencing using a forward and reverse fusion primer. The forward primer was constructed with (5'-3') the Roche A linker (CCATCTCATCCCTGCGTGTCTCCGACTCAG), an 8-10 bp barcode, and the forward primer pnp349F for *pnpA* and mar349BF for *mar* gene fragment amplification (**Table 2.3**). The reverse fusion primer was constructed with (5'-3') a biotin molecule, the Roche B linker (CCTATCCCCTGTGTGCCTTGGCAGTCTCAG), and the primer pnp635R for *pnpA* and mar715AR for *mar* gene fragment amplification (**Table 2.3**). Amplifications were performed in 25 μ L reactions with Qiagen HotStart Taq master mix (Qiagen Inc, Valencia, California), 1 μ L of each 5 μ M primer, and 1 μ L of template. Reactions were performed on ABI Veriti thermocyclers (Applied Biosystems, Carlsbad, California) under the following thermal profile; 95 °C for 5 minutes, then 35 cycles of 94 °C for 30 seconds, 54 °C for 40 seconds, 72 °C for 1 minute, followed by one cycle of 72 °C for 10 minutes and a 4 °C hold.

Amplification products were visualized with eGels (Life Technologies, Grand Island, New York). Products were then pooled equimolar and each pool was cleaned with Diffinity RapidTip (Diffinity Genomics, West Henrietta, New York), and size selected using Agencourt AMPure XP (BeckmanCoulter, Indianapolis, Indiana) following Roche 454 protocols (454 Life Sciences, Branford, Connecticut). Size selected pools were then quantified and 150 ng of DNA were hybridized to Dynabeads M-270 (Life Technologies) to create single stranded DNA following Roche 454 protocols (454 Life Sciences). Single stranded DNA was diluted and used in emPCR reactions, which were performed and subsequently enriched. Sequencing

following established manufacture protocols (454 Life Sciences) using Roche 454 FLX instrument with Titanium reagents, titanium procedures performed at the Research and Testing Laboratory (Lubbock, TX) (RTL, 2012).

2.3.17. Analysis of 454 sequence data

The high throughput data was analysed using the Quantitative Insight into Microbiology (Qiime) pipeline according to Caporaso *et al.* (2010 a). The obtained sequences for *pnpA* and *mar* genes, from triplicates of T₀ and T_{end} samples, were used to trim the barcodes using the default pipeline. Operational Taxonomic Units (OTUs) were picked at 95% similarity of DNA sequences for clustering, using `pick_otus.py` according to Edgar (2010). Relative abundance of sequences forming OTUs was calculated in the samples. Sequences below 1% abundance were discarded since they mainly represented singletons and did not contribute significantly to the total number of sequences. The representative sequences from remaining OTUs were analysed using an NCBI BLAST search (Altschul *et al.*, 1990) and those that were not *pnpA* or *mar* were excluded from downstream analysis. To assign taxonomy to picked OTUs, a taxonomy information mapping file as well as a fasta file with representative sequences for functional genes was created based on the previously described clone libraries from the ARB database for both functional genes *pnpA* and *mar*. RDP Classifier 2.2. was used according to Wang *et al.* (2007). The re-train RDP option was applied to assign taxonomy to representative OTUs for both functional genes. The representative DNA sequences from OTUs were aligned using the default Qiime pipeline according to Caporaso *et al.* (2010 b) using Multiple Sequence Comparison by Log-Expectation (MUSCLE), alignment software which was chosen as an option available in the pipeline. Alignment was filtered and alpha diversity was analysed using default settings of the Qiime pipeline. Representative sequences from OTUs were uploaded to the *pnpA* and *mar* ARB database and translated and aligned as described above. Representative sequences from OTUs were added to existing neighbour-joining phylogenetic trees with *pnpA* and *mar* reference sequences and sequences derived from cloning.

2.3.18. Statistical analyses

2.3.18.1. Statistical analysis of PNP biodegradation and QPCR data

Analysis of variance (ANOVA) was used to identify the significance of differences ($P < 0.05$) in PNP biodegradation and the associated microbial communities between treatments. Based on the HPLC measurements for PNP biodegradation time to 15, 50 and 90% PNP-degradation (DT_{15} , DT_{50} , and DT_{90}), the length of the lag phase and maximum PNP biodegradation rates were calculated for triplicates of light and dark incubated river water, using the model of best fit to the biodegradation kinetics which was determined for each sample, as described by Rodriguez-Cruz *et al.* (2006). ANOVA was also used to determine the significance of differences in the number of PNP degrading bacteria (one-way ANOVA) and the number of copies of functional genes *pnpA* and *mar* (two-way ANOVA) from triplicates of treatments before and after PNP biodegradation. The least significant difference (LSD) was used to found the significant differences between treatments. All statistical analyses were performed using GenStat (13th edition, VSN International Ltd.).

2.3.18.2. Statistical analysis of bacterial community diversity using TRFLP profiles

Traces were transferred from GeneMarker to Microsoft Excel and the total number of TRFs along with the total trace intensity was calculated. Following this, any TRFs that were found to represent less than 1% of the total intensity of a trace were discarded and recorded as “0” (MacDonald *et al.*, 2007). The three replicates of each sample were pooled to produce an average TRFLP profile for each treatment (Lukow *et al.*, 2000). The first stage of analysis was to input the data into the programme as abundance data (not transformed), and to then carry out a resemblance analysis on it using the Bray-Curtis dissimilarity formula. This formula is used to compare the compositional differences between two different data sets (e.g. two individual TRFLP traces). The analysis uses the formula detailed below:

$$BC_{ij} = \frac{S_i + S_j - 2C_{ij}}{S_i + S_j}$$

where C_{ij} is the sum of the minimum abundances of each data point (e.g. individual TRFs) across two different data sets; and S_j and S_i are the total number of data points (e.g. total TRF numbers) across the two different data sets (Bray and Curtis, 1957). Principal component analysis was performed for bacterial TRFLP profiles using Primer 6 software V10.1.12 (PRIMER-E Ltd. UK), where the abundance of TRFs was compared between treatments. Resemblance analysis was followed by non-metric multidimensional scaling (NMDS). Coordinates were used to create the NMDS plots. Analysis of similarities (ANOSIM) was also performed for TRFLP profiles based on calculated resemblance to find the significance of similarities between TRFLP profiles. Clustering and 2D bubble graphs were performed using Primer 6 to study the similarities between bacterial TRFLP profiles and the abundance of chosen TRFs, respectively. The TRF 245 nt was chosen for the analysis since it was predominant in TRFLP profiles following the biodegradation of PNP. This TRF was also identified as characteristic for the PNP-degrading *Pseudomonas syringae* isolate AKHD2 based on TRFLP profile from that isolate. Relative abundance of the TRF 245 nt was calculated using Microsoft Excel, and expressed as a percentage of fluorescence intensity of that TRF peak relative to the sum of fluorescence intensity of all TRF peaks present in bacterial profiles. ANOVA was calculated for the abundance of TRF 245 nt in bacterial profiles from river water using GenStat (13th edition, VSN International Ltd.).

2.3.18.3. Statistical analysis of the PNP degrading communities using *pnpA* and *mar* sequences

Populations of *pnpA* and *mar* sequences obtained from clone libraries from pooled triplicates of T_0 and PNP degrading river water (HD) were grouped using 95% sequence similarity. For comparing clone libraries from T_0 and after PNP biodegradation in the dark (HD), the impacts on diversity were analysed using a Chao1 estimate of population size, including a calculation of the Chao1 variance to determine whether any changes are significant (Chao, 1987). The Chao1 estimate of population size was calculated as follows:

$$S = D + \frac{f_1(f_1-1)}{2(f_2+1)}$$

Where S is the Chao1 estimate of the actual population size, D is the observed population size (i.e. the number of categories) and f₁ and f₂ are the number of categories which are represented by 1 and 2 individuals respectively. Chao1 variance can then be calculated using the formula:

$$\text{Chao1 variance} = (f_2+1)(0.25R^4 + R^3 + 0.5R^2), \text{ where } R = (f_1-1)/(f_2+1)$$

A Mann-Whitney U-test was used to determine whether there was a significant difference in the community structure of *pnpA* and *mar* clone libraries from T₀ and HD river water. For this analysis the samples (clones) in each library, separately from T₀ (population 1) and HD samples (population 2), were first ranked from highest to lowest according to the proportion of the population they represent. The n₁ and n₂ values were derived and the U value was calculated for each clone library as follows:

$$U = (n_1 \times n_2) - (n_1(n_1+1))/2 - R_1$$

Where n₁ and n₂ are the numbers of samples in populations 1 and 2 and R₁ is the sum of the ranks for the samples in population 1. The data values for U, n₁ and n₂ were then entered into the Mann-Whitney U-Test page of the *Caenorhabditis elegans* (2011), where the probability value for U was calculated and the two populations compared.

2.4. Results

2.4.1. The biodegradation of PNP

The biodegradation of PNP was recorded within eight days for river water incubated in the dark with 2 mg/L of PNP (**Figure 2.4**). Under light conditions, only one replicate (n=3) of river water with PNP at 2 mg/L degraded PNP after a much longer adaptation phase (17 days) than in the dark incubated river water. No PNP biodegradation was recorded for river water incubated under dark and light conditions with PNP at 20 µg/L, and HPLC analysis was affected by the detection limit for PNP which was just below 20 µg/L. Due to the background interference from river water samples, analysis of PNP in the treatments with low concentrations was difficult and considered unreliable. Incubation of these treatments was finished at day eight, and chromatograms had a peak present at the same retention time as the PNP peak. The PNP biodegradation curves obtained for dark incubated river water were similar, and the statistical analysis revealed significant differences in PNP biodegradation between light and dark incubated river water.

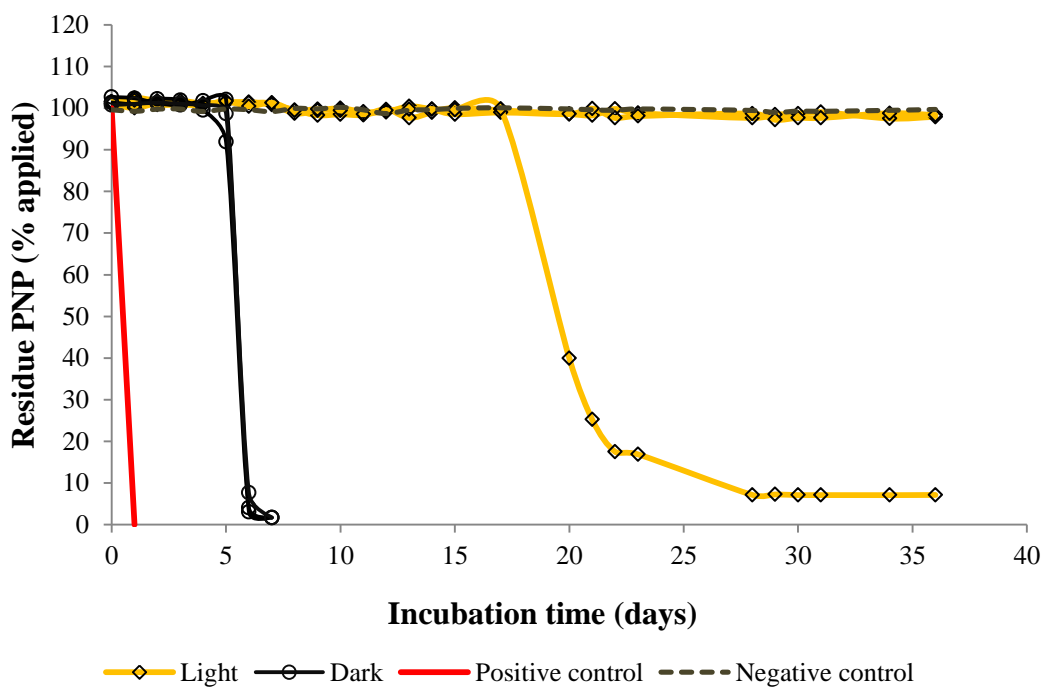


Figure 2.4: The biodegradation of PNP under light and dark conditions.

The values for DT_{15} , DT_{50} , DT_{90} (**Table 2.4**) were calculated for treatments which degraded PNP. The single replicate of light incubated river water which degraded PNP at 2 mg/L had DT_{15} , DT_{50} and DT_{90} values at 18, 19.5 and 27.5 days, respectively.

Table 2.4: The DT-values for PNP biodegradation in river water under dark conditions.

Sample	DT_{15} (days)	DT_{50} (days)	DT_{90} (days)
High PNP + Dark	4.57 (± 0.54)	5.19 (± 0.31)	5.90 (± 0.05)

Where: \pm standard error of the mean (S.E.M.) in brackets; n=3

2.4.2. Inoculum size

Initial inoculum determined by plating river water aliquots on R2A agar was 3.68×10^4 cfu/mL (n=1).

2.4.3. Flow cytometry measurements

Flow cytometry measurements (n=1) revealed that the number of bacteria and algae was 8.67×10^4 and 1.62×10^4 cfu/mL, respectively in T_0 river water. The number of bacteria increased after PNP biodegradation in the dark to 3.45×10^5 cfu/mL in the bottles with high PNP concentration (2 mg/L), but the number of algae decreased to 8.24×10^3 cfu/mL. Bottles with low PNP (20 μ g/L) incubated under dark conditions had lower numbers of bacteria and algae (3.86×10^3 and 9.11×10^3 cfu/mL). Both, the number of bacteria and algae increased under light conditions to 3.45×10^5 and 4.38×10^5 ; 2.22×10^5 and 6.53×10^5 cfu/mL (n=1) in bottles with high and low PNP, respectively.

2.4.4. Isolation and identification of PNP degraders

Following the procedure described in section 2.3.5.2 bacterial strains degrading PNP were isolated and identified. Total number of detected PNP degrading bacteria was 1.19×10^5 cfu/mL ($\pm 3.71 \times 10^4$; n=2) in dark incubated river water degrading PNP. Strains capable of PNP biodegradation (**Table 2.5**) were isolated from river water after biodegradation of 2 mg/L PNP under dark conditions (isolate AKHD1, AKHD2, AKHD3) and from river water incubated with 20 µg/L of PNP in the dark (with no PNP biodegradation) (isolate AKLD1 and AKLD2). Using the NCBI database 16S rRNA gene sequences were analysed using blastn against the Genbank database (Altschul *et al.*, 1990). One strain was closely related to the genus *Bacillus*, the other four were related to *Pseudomonas*. PCR amplification of functional genes from DNA extracts obtained from bacterial isolates indicated the presence of the *pnpA* gene in *Pseudomonas fluorescens*-like and *Pseudomonas syringae*-like isolates. The *npdA2* gene was amplified only from the *Bacillus pumilus* DNA extract. *mar* gene was present in all isolates. Additionally, the biodegradation of PNP carried out in liquid mineral medium using ^{14}C PNP assay revealed that only *Pseudomonas syringae* AKHD2 was capable of PNP biodegradation at the high PNP concentration (2014 µg/L) (**Figure 2.5 b**). No biodegradation was observed at the low PNP concentration (14 µg/L) even for the control strain *Arthrobacter* sp. JS443 (**Figure 2.5 a**).

Table 2.5: Properties of bacterial isolates.

Isolate	16S rRNA closest relation in Genbank (accession number)	%ID	Halo on MM+PNP plate	PNP biodegradation in liquid MM	Presence of <i>pnpA</i> gene	Presence of <i>npdA2</i> gene	Presence of <i>mar</i> gene
AKLD1	<i>Pseudomonas fluorescens</i> Pf0-1 (NC_007492.2)	98	+	-	+	-	+
AKLD2	<i>Bacillus pumilus</i> ATCC 7061 (NZ_ABRX01000003.1)	99	+	-	-	+	+
AKHD1	<i>Pseudomonas fluorescens</i> SBW25 (NC_012660.1)	99	+	-	+	-	+
AKHD2	<i>Pseudomonas syringae</i> (ACXT01000186.1)	98	+	+	+	-	+
AKHD3	<i>Pseudomonas syringae</i> (ACXT01000186.1)	98	+	NM	+	-	+

NM- not measured

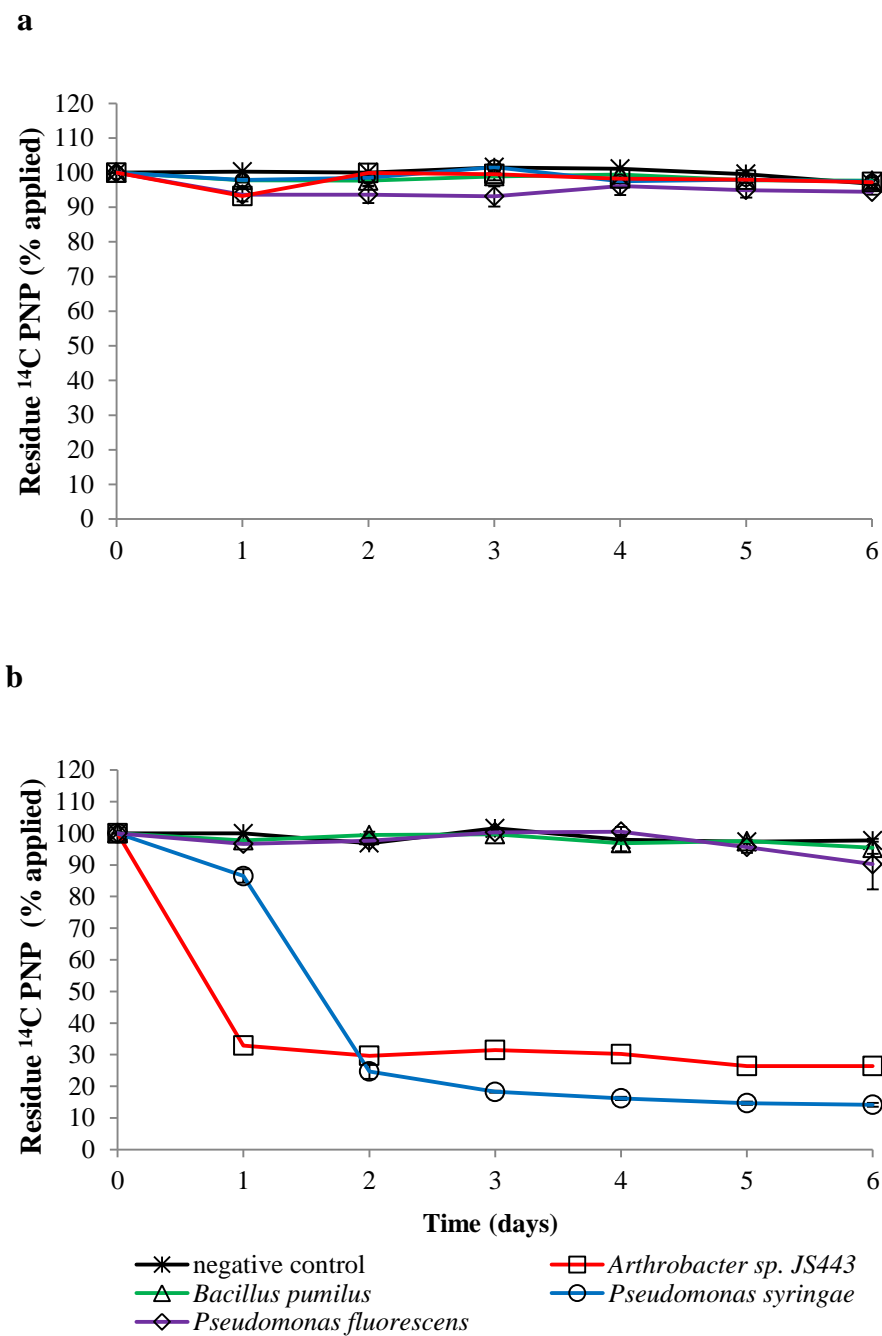


Figure 2.5: The biodegradation of PNP with bacterial isolates.

Figure legend: **a**- ^{14}C PNP biodegradation at a total PNP concentration of $14\ \mu\text{g/L}$; **b**- ^{14}C PNP biodegradation at a total PNP concentration of $2014\ \mu\text{g/L}$; error bars are showing standard error of the mean (S.E.M.), $n=3$.

2.4.5. pH measurement

Significant changes ($P < 0.001$) to water pH were observed in light bottles (**Table 2.6**) in comparison to pH measured in freshly collected river water and river water incubated under dark conditions. Water pH increased in light bottles and possibly inhibited PNP biodegradation. The pH may have increased due to the algal assimilation of dissolved CO_2 (carbonic acid) from water during the process of photosynthesis.

Table 2.6: Changes in river water pH after incubation period of eight and 36 days in the dark and light, respectively.

Treatment	pH	
	Before incubation (T_0)	After eight and 36 days of incubation in the dark and light, respectively
Dark + High PNP	8.14 (± 0.02)	8.06 (± 0.02) ^a
		8.17 (± 0.02) ^a
		8.08 (± 0.01) ^a
Dark + Low PNP	8.14 (± 0.02)	8.03 (± 0.02) ^a
		8.05 (± 0.03) ^a
		8.08 (± 0.03) ^a
Light + High PNP	8.14 (± 0.02)	10.12 (± 0.12) ^b
		9.83 (± 0.06) ^c
		10.24 (± 0.06) ^b
Light + Low PNP	8.14 (± 0.02)	9.52 (± 0.13) ^d
		9.79 (± 0.03) ^c
		9.34 (± 0.05) ^d

Where: treatments with different letters are significantly different ($P < 0.05$); \pm standard error of the mean (S.E.M.) in brackets; $n=3$.

2.4.6. Direct effect of light and pH on the biodegradation of PNP

The biodegradation of PNP with isolate *P. syringae* occurred under both light and dark incubation in mineral medium at pH 7.5, and therefore, light had no direct impact on the biodegradation of PNP. Inhibition of PNP biodegradation was observed under light and dark conditions in liquid medium at pH 9.8 (**Figure 2.6 a and b**).

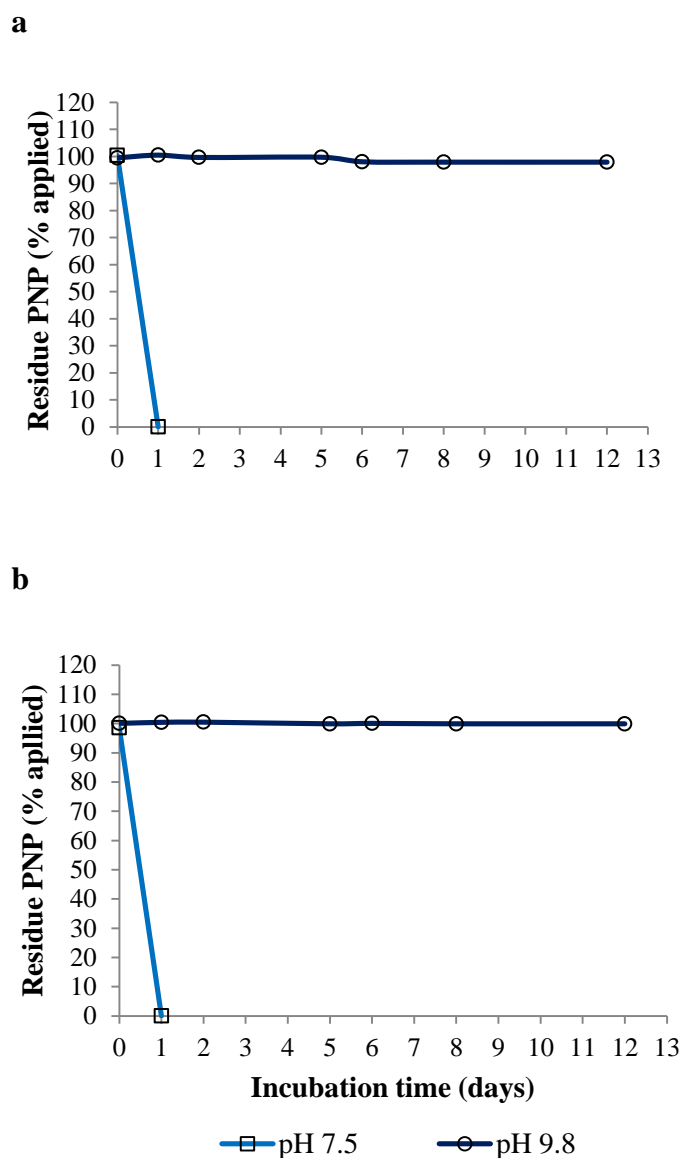


Figure 2.6: The biodegradation of PNP under a- light and b- dark conditions in mineral medium with low pH 7.5 and high pH 9.8; (n=1).

2.4.7. Bacterial community profile using TRFLP

The NMDS analysis (**Figure 2.7 a**), performed with Primer 6 (version 6.1.12), showed an increasing similarity from 40 to 60% between T₀ bacterial TRFLP profiles and profiles of river water after PNP biodegradation at 2 mg/L in the dark (Examples of TRFLP profiles are given in **Appendix Figure 0.5**.) River water incubated in the dark and light with 20 µg/L of PNP had TRFLP profiles with much lower similarity (40%) between replicates. Similarity between replicates of light incubated river water with 2 mg/L PNP was also low (< 20%) and these profiles clustered separately from other treatments (**Figure 2.7 b**). The ANOSIM revealed significant differences between TRFLP profiles for river water before the incubation (T₀) and river water incubated with 2 mg/L of PNP in the light and dark. Incubation conditions and the presence of PNP at high concentration were the main factors responsible for changes in river water bacterial TRFLP profiles. The presence of PNP at lower (20 µg/L) concentration did not affect the bacterial TRFLP profiles under both dark and light conditions.

The TRFLP analysis of 16S rRNA genes was performed for the isolates obtained from dark incubated river water, strains *Pseudomonas syringae*, *Pseudomonas fluorescens* and *Bacillus pumilus*. Interestingly, TRFLP analysis of 16S rRNA gene from *Pseudomonas syringae* AKHD2, resulted in the same TRF of 245 nt which was seen as increasing in abundance at the higher PNP concentration in dark incubated river water replicates after PNP biodegradation. The amount of the *P. syringae* TRF 245 nt presented in **Figure 2.8** confirmed the abundance and proliferation of *P. syringae* and probably other *Pseudomonas*-like bacteria in the dark incubated river water degrading PNP and that this PNP degrader was also present in the river water before incubation. TRFLP analysis also suggested that it was absent from light incubated treatments with high PNP concentration (**Table 2.7**) and that the TRF of 245 nt had a significantly lower abundance (P<0.001) in light and dark incubated river water with low PNP concentration. Identification of other TRFs was not performed.

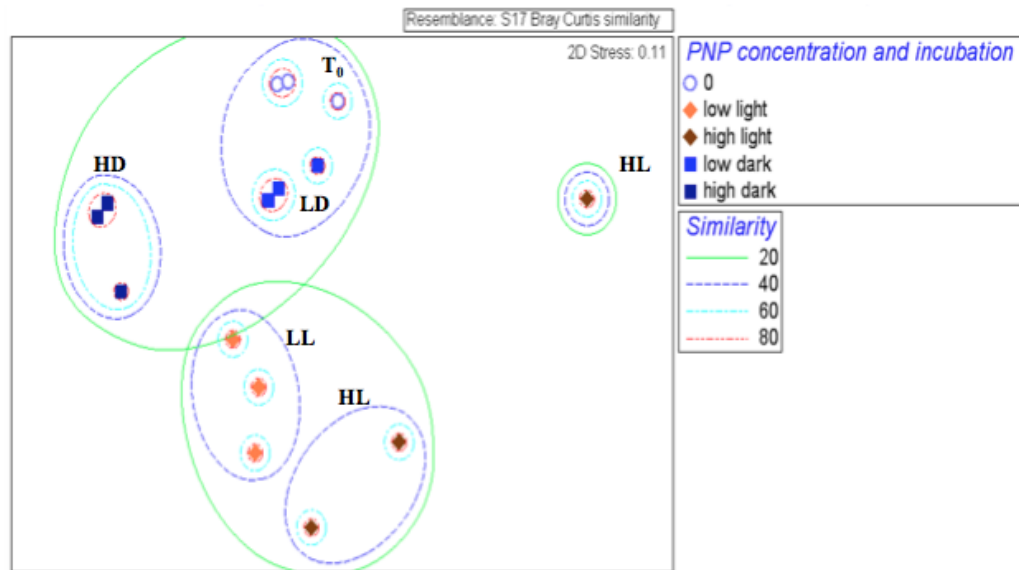
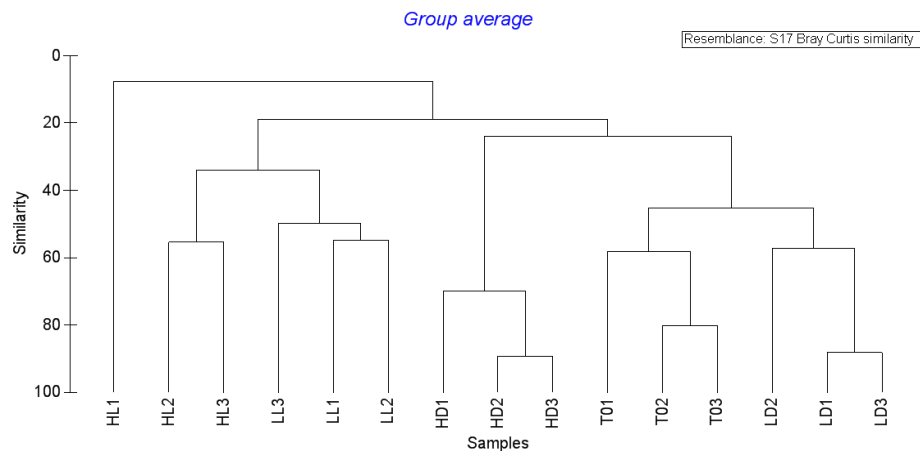
a**b**

Figure 2.7: a- Non-Metric Multidimensional Scaling (NMDS) plot of TRFLP bacterial profiles, b- dendrogram showing similarities between TRFLP profiles and Bray-Curtis distance for the data set derived from amplification with bacterial primers 63F and 1087R-VIC fluorescently labelled, and digested with *MspI* and *HhaI*. Stress 0.11. Where T_0 - freshly collected river water; **LL1-3-replicates of light incubated river water with low PNP; **HL1-3**- replicates of river water incubated in the light with high PNP; **LD1-3**- replicates of river water incubated in the dark with low PNP; **HD1-3**- replicates of river water incubated in the dark with high PNP; high and low PNP concentration was 2 mg/L and 20 μ g/L, respectively.**

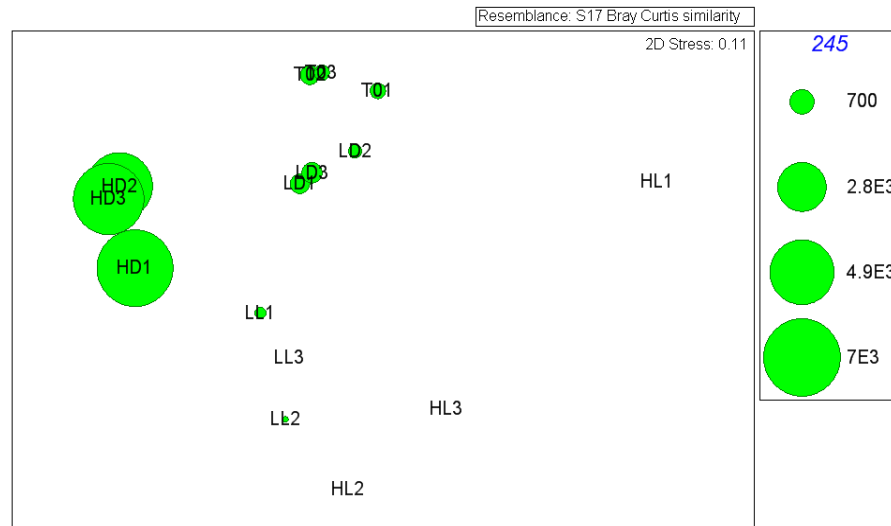


Figure 2.8: A 2D bubble plot presenting the amount of TRF 245 nt (TRF identified as *Pseudomonas syringae*) in environmental samples using Bray-Curtis similarity. Stress 0.11. Figure legend: **T₀- freshly collected river water; **LL1-3**-replicates of light incubated river water with low PNP; **HL1-3**- replicates of river water incubated in the light with high PNP; **LD1-3**- replicates of river water incubated in the dark with low PNP; **HD1-3**- replicates of river water incubated in the dark with high PNP; high and low PNP concentration was 2 mg/L and 20 µg/L, respectively.**

Table 2.7: Relative abundance of TRF 245 nt in bacterial TRFLP profiles of river water.

Incubation	PNP concentration treatment	Relative abundance of TRF 245 nt (%)
T₀	-	8.51 (± 1.66) ^a
Dark	Low	12.29 (± 0.61) ^a
	High	51.33 (± 4.75) ^c
Light	Low	1.16 (± 0.89) ^b
	High	0.00 (± 0.00) ^b

Where treatments with different letters are significantly different $P < 0.05$; ± standard error of the mean (S.E.M.) in brackets; $n=3$.

2.4.8. QPCR for *pnpA* and *mar*

Number of copies of both markers was determined in the environmental samples before and after light and dark incubation of river water with high and low PNP concentration (**Figure 2.9 a and b**). ANOVA showed that the number of copies of both genes *pnpA* and *mar* increased significantly ($P < 0.001$ for *pnpA* and for *mar*) after 2 mg/L PNP biodegradation in dark incubated river water (HD), while no differences were recorded for the number of copies of either marker in light incubated river water. Also, dark incubated river water with 20 µg/L PNP (LD) had lower number of copies of both markers. Surprisingly, a higher copy number was found for the *pnpA* gene compared to *mar* in all treatments. Statistical analysis using ANOVA for the number of copies of 16S rRNA gene revealed that a higher copy number of the 16S rRNA gene was present in T_0 river water, dark incubated river water which degraded 2 mg/L PNP and in light incubated river water with high and low PNP concentration (**Figure 2.9 c**). River water incubated in the dark with 20 µg/L PNP (LD) had significantly lower copy number of 16S rRNA gene.

It is noteworthy that non-target hits are present in the copy number for *pnpA*, and *mar* copy number does not represent PNP-degraders only. Also, 16S rRNA gene copy number is low (possibly due to the long gene fragment) which may suggest that counts do not represent the actual abundance of bacteria in the samples.

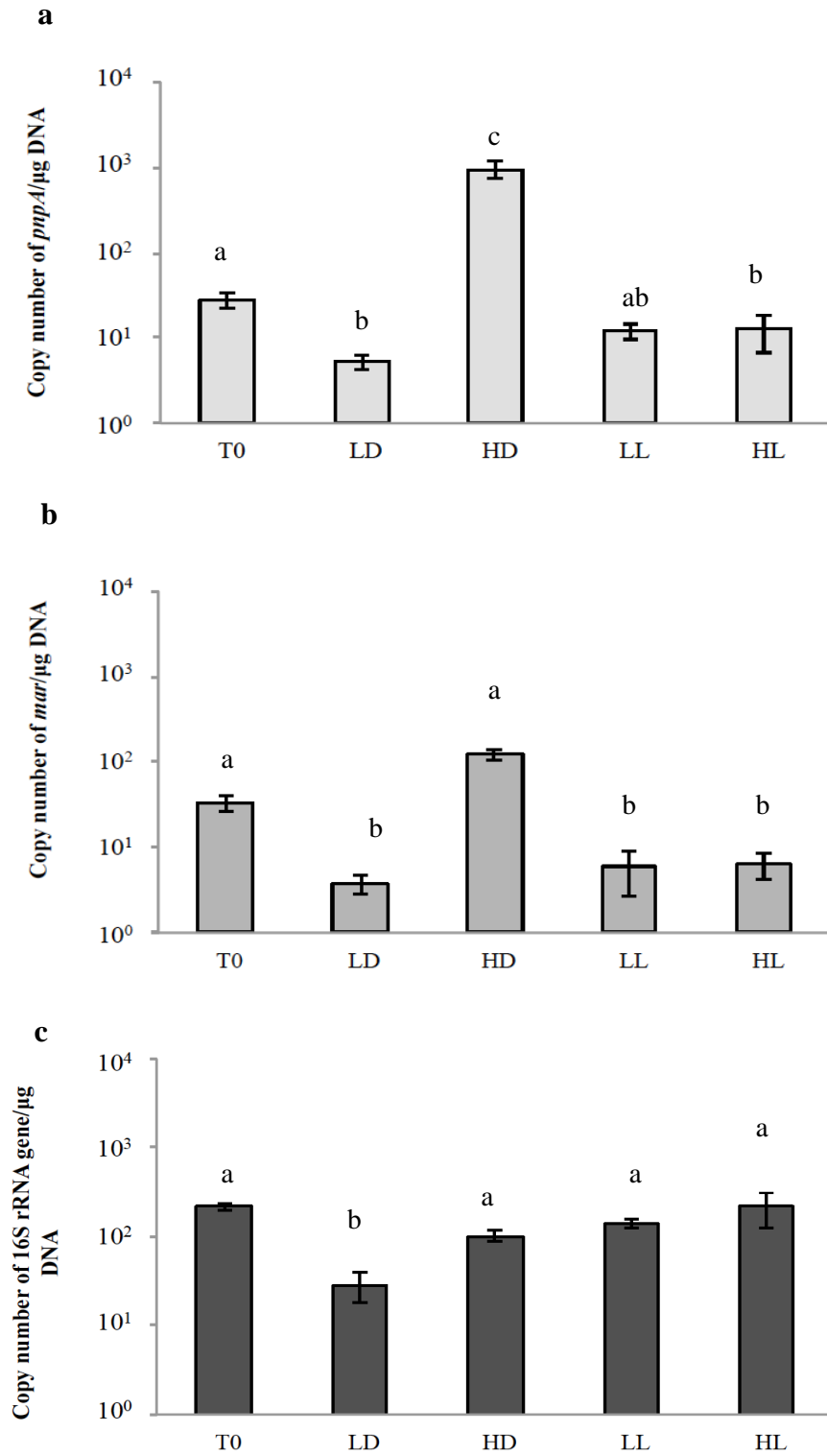


Figure 2.9: QPCR for functional genes and 16S rRNA gene.

Figure legend: **a**- *pnpA* and **b**- *mar* and **c**-16S rRNA; where **T₀**- freshly collected river water; **LD**- river water incubated under dark with low PNP; **HD**- river water incubated under dark with high PNP; **LL**- river water incubated under light with low PNP; **HL**- river water incubated under light with high PNP. Treatments with different letters are significantly different ($P < 0.05$); error bars show \pm standard error of the mean (S.E.M.), $n=3$.

2.4.9. Functional marker analysis

To check the potential diversity of PNP degrading bacteria, PNP functional marker analysis was performed as described above. The PNP degradative genes *pnpA*, *npdA2* and *mar* were targeted with designed primers and amplified from environmental samples. While *pnpA* and *mar* were detected, no products were obtained for *npdA2* in river water at T₀ or after complete PNP biodegradation. Clone libraries were constructed using sequences for *pnpA* and *mar* genes from T₀ river water and water degrading high PNP concentration in the dark. Additionally, *pnpA* and *mar* were amplified from isolate *Pseudomonas syringae* AKHD2, previously identified as a PNP degrading bacterium.

2.4.9.1. Sequencing and clone library for *pnpA*

The *pnpA* functional marker analysis revealed that two main clades (**Figure 2.10**) of *pnpA* gene sequences were present in environmental samples before and after incubation with PNP and clones from each treatment differentially contributed to each of those clades. The main clades were characterized as *Pseudomonas syringae*-clade 1 with sequences from *P. syringae* AKHD2, and clade 2 with sequences from previously undetected *Pseudomonas*-like bacteria. The number of successful *pnpA* clones was 37 and 42 for T₀ and HD samples, respectively out of each 48 clones sequenced. Chao 1 analysis was carried out for *pnpA* gene clone library to determine if the biodegradation of 2 mg/L PNP in the dark had a significant impact on river water bacterial diversity. Chao 1 estimate of diversity sampled indicated that 41 and 55% of the predicted total diversity of *pnpA* gene sequences had been represented within clone library for T₀ and HD samples, respectively. The Mann-Whitney U-test showed that overall there was no significant difference in *pnpA* gene community structure from T₀ and PNP degrading river water (P=0.066).

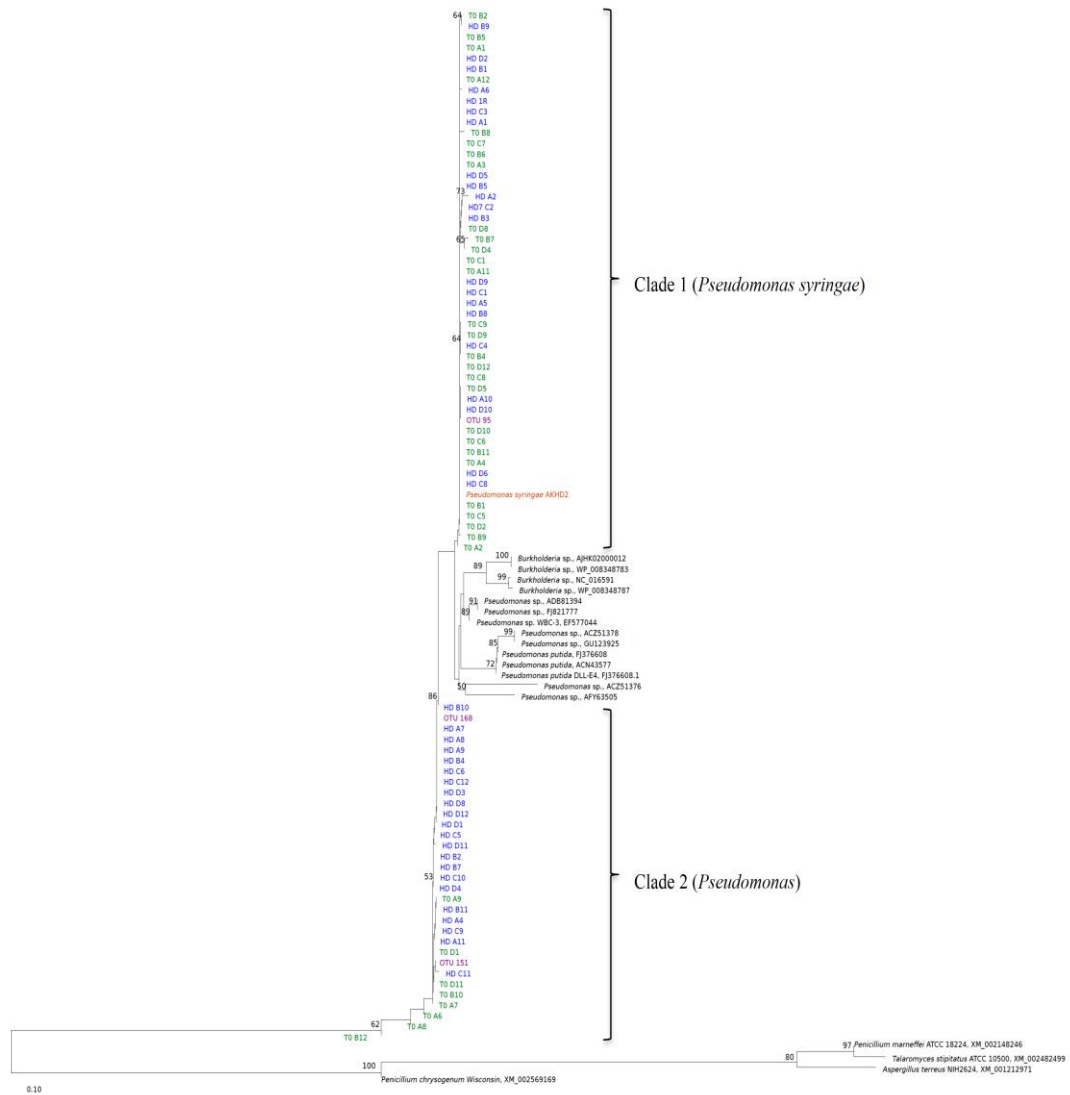


Figure 2.10: Neighbour-joining tree showing diversity of *pnpA* gene sequences obtained from cloning and 454 sequencing. Tree created based on nucleotide sequences translated into amino acid sequences using ARB (Ludwig *et al.*, 2004). Figure legend: **in black**- reference sequences followed by accession numbers; **in green**- sequences obtained from clone library from T₀ samples; **in blue**- sequences obtained from clone libraries from HD samples; **in orange** – *pnpA* sequence from *Pseudomonas syringae* isolate AKHD2; **in pink**- representative *pnpA* sequences from OTUs obtained from 454 sequencing from both T₀ and HD samples. The scale bar represents 10% sequence divergence. Numbers on each branch show the bootstrap value of 100 replicates (expressed as %). Branches with no values have less than 50 % bootstrap support.

2.4.9.2. Sequencing and clone library for *mar*

A total of 48 *mar* clones were sequenced from each T₀ and HD samples. 24 *mar* gene sequences were from T₀ river water and 38 *mar* gene sequences were from dark incubated river water degrading PNP. In total 62 new *mar* gene sequences were included in the tree used to design primers for *mar* gene. Seven different clades were identified and an additional 11 single *mar* sequences grouped separately (not within clades). Analysis of *mar* functional marker showed a higher number of clades (**Figure 2.11**) within the *mar* clone library, suggesting that a higher diversity of potential PNP degraders was detected using this gene compared to the *pnpA* clone library. Sequences of *mar* gene grouped not only within γ -Proteobacteria such as *Pseudomonas syringae* but also within α -Proteobacteria (e.g. *Rhizobium leguminosarum* bv. *viciae* 3841), β -Proteobacteria (e.g. *Burkholderia cenocepacia* J2315, *Burkholderia multivorans*) and Actinobacteria (e.g. *Rhodococcus opacus* RHA1). Additionally, proliferation of taxa grouping within clades containing *Rhizobium leguminosarum* bv. *viciae* 3841, *Agrobacterium vitis* S4, *Bordetella petrii* and *Pseudomonas syringae* AKHD2 was observed in river water degrading PNP (**Table 2.8**) .

Chao 1 analysis was carried out as described above. Chao 1 estimate of diversity sampled indicated that 23 and 11% of the predicted total diversity of *mar* sequences had been represented within the clone library for T₀ and HD samples, respectively. The Mann-Whitney U-test revealed a significant difference ($P < 0.0001$) in *mar* gene community structure from T₀ and PNP degrading river water.

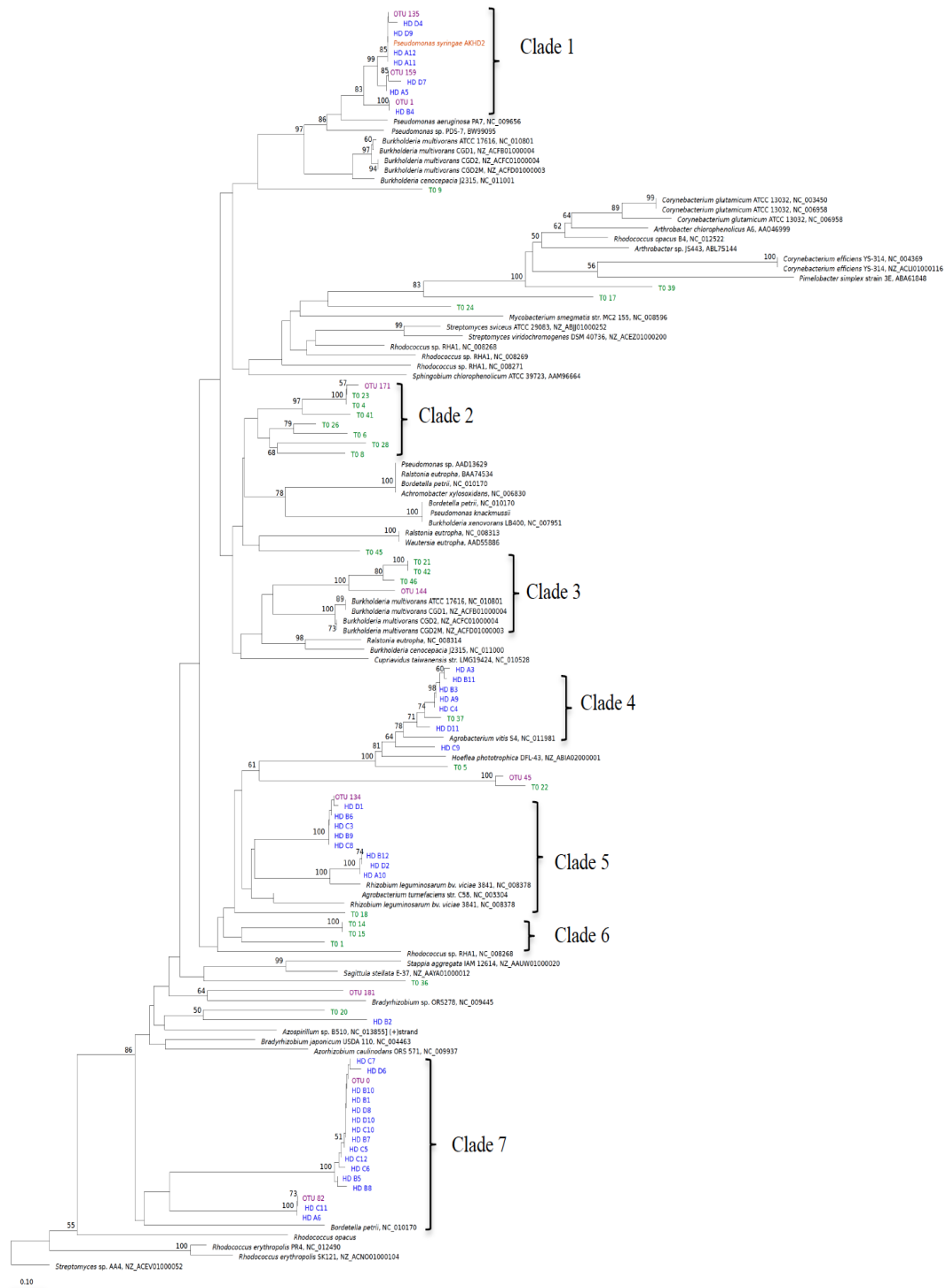


Figure 2.11: Neighbour-joining tree showing diversity of *mar* gene sequences obtained from cloning and 454 sequencing. Tree created based on nucleotide sequences translated into amino acid sequences using ARB (Ludwig et al., 2004). Figure legend: **in grey**- reference sequences followed by accession numbers; **in green**- sequences obtained from clone library from T₀ samples; **in blue**- sequences obtained from clone libraries from HD samples; **in orange** – *mar* sequence from *Pseudomonas syringae* isolate AKHD2; **in pink**- representative *mar* sequences from OTUs obtained from 454 sequencing from both T₀ and HD samples. Representative sequences for OTU 8, 99 and 104 were excluded from the *mar* alignment due to poor sequence quality. The scale bar represents 10% sequence divergence. Numbers on each branch show the bootstrap value of 100 replicates (expressed as %). Branches with no values have less than 50 % bootstrap support.

Table 2.8: Diversity of *mar* clones from T₀ and PNP degrading river water based on *mar* neighbour-joining tree. 86% similarity at amino acid level was used as a cutoff to distinguish clades within a preexisting tree.

Closest related clade in phylogenetic tree	Clade number	% <i>mar</i> clones	
		T ₀	Dark
<i>Pseudomonas syringae</i> AKHD2	1	0	18.4
<i>Burkholderia cenocepacia</i> J215	single <i>mar</i> sequence	4.2	0
<i>Pimelobacter simplex</i> strain 3E	single <i>mar</i> sequence	4.2	0
<i>Mycobacterium smegmatis</i> strain MC2	single <i>mar</i> sequence	8.3	0
<i>Achromobacter xylosoxidans</i>	2	29.2	0
<i>Wautersia eutropha</i>	single <i>mar</i> sequence	4.2	0
<i>Burkholderia multivorans</i>	3	12.5	0
<i>Agrobacterium vitis</i> S4	4	4.2	18.4
<i>Hoeflea phototrophica</i> DFL-43	single <i>mar</i> sequences	8.3	0
<i>Rhizobium leguminosarum</i> bv. <i>vitiae</i> 3841	5	4.2	21.1
<i>Rhodococcus</i> sp. RHA1	6	12.5	0
<i>Sagittula stellata</i> B-37	single <i>mar</i> sequence	4.2	0
<i>Azospirillum</i> sp. B510	single <i>mar</i> sequences	4.2	2.6
<i>Bordetella petrii</i>	7	0	39.5

2.4.10. 454 sequencing for PNP functional genes

Since no PNP biodegradation could be detected at low PNP concentration and PNP biodegradation was inhibited under light conditions, the functional gene diversity was determined for river water which degraded a high PNP concentration in the dark, and this was compared with the diversity of degraders present in T₀ river water. Since Chao 1 and collector curve analysis of clone libraries data suggested incomplete coverage of the diversity, high throughput sequencing was used in addition to determine in depth the diversity of PNP functional markers in the river water.

2.4.10.1. 454 for *pnpA*

The total number of reads is presented in **Appendix Table 0.4**. The majority of 454 reads represented amplification of non-targeted genes that are not involved in PNP biodegradation pathways (false positives) e.g. alcohol dehydrogenase, haloacid dehalogenase, and some hypothetical proteins. Those reads were discarded and not included in the analysis. In total, 65,459 sequences were obtained and 40,463 *pnpA* sequences were used for downstream analysis. Those sequences clustered within three OTUs picked from T₀ river water and river water samples degrading high PNP concentration in the dark (HD). Difficulties with *pnpA* amplification from T₀ river water samples resulted in much lower number of sequences from those samples, and therefore, they were not included in the analysis for alpha diversity. Similar diversity was observed for HD samples.

The OTU profiles for T₀ and HD samples (OTUs formed by more than 1% of total *pnpA* sequences per sample) were compared (**Figure 2.12**). The sequences from T₀ samples were not included since they represent very low percentage of total number of *pnpA* sequences. *pnpA* sequences from HD1 sample contributed to OTU 95, sequences from HD2 samples formed all three OTUs (95, 151 and 168) and sequences present in sample HD3 contributed to OTUs 95 and 168. Overall, variation in *pnpA* diversity was observed between replicates within treatments.

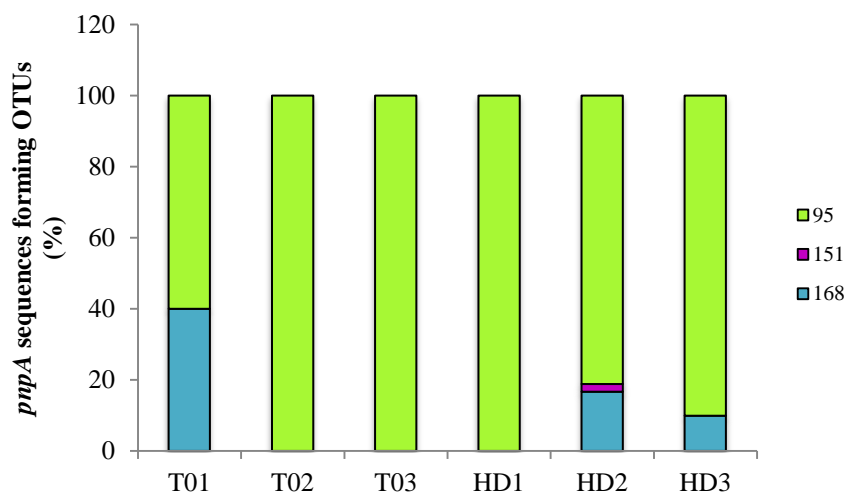


Figure 2.12: The relative abundance of *pnpA* sequences as % of total *pnpA* sequences forming OTUs from T_0 and river water degrading high PNP concentration in the dark (HD). Where: OTU numbers are given in the figure legend, T_0 1- T_0 3 –replicates of freshly collected river water, HD1-HD3- replicates of river water degrading high PNP concentration in the dark.

Representative *pnpA* sequences from three OTUs were analysed using BLAST search (NCBI). OTUs present mainly in the dark river water which degraded PNP, OTU 95, 151 and 168 (**Table 2.9**), were represented by *pnpA* sequences which resulted in the closest relation to *pnpA* from *Pseudomonas* sp. WBC-3. OTUs 95 and 168 consisted of *pnpA* sequences from both, T_0 and dark incubated river water degrading PNP. Sequences from dark incubated river water degrading PNP contributed mostly to picked OTUs.

Table 2.9: Identity and relative abundance of *pnpA* gene sequences in picked OTUs from T_0 river water and water degrading high PNP concentration in the dark (HD).

Sample	OTU No.	% total sequences	BLAST results (accession number)	% ID	References
T_0 + HD	95	92.64	<i>para</i> -nitrophenol 4-monooxygenase <i>Pseudomonas</i> sp. WBC-3 (ABU50908.1)	90	Zhang <i>et al.</i> , 2009
HD	151	0.52	<i>para</i> -nitrophenol 4-monooxygenase <i>Pseudomonas</i> sp. WBC-3 (ABU50908.1)	85	
T_0 + HD	168	6.84	<i>para</i> -nitrophenol 4-monooxygenase <i>Pseudomonas</i> sp. WBC-3 (ABU50908.1)	86	

Using the ARB database and alignment for *pnpA* sequences, representative sequences from OTUs detected by high throughput sequencing were translated from DNA to amino acid sequences, aligned and included in the phylogenetic tree (**Figure**

2.10). There was low phylogenetic diversity between picked OTUs. OTU 95 clustered within clade 1 which was previously described as *P. syringae* clade, while OTUs 151 and 168 grouped within clade 2 (undetected *Pseudomonas*).

2.4.10.2. 454 for *mar*

In total, 22,318 sequences were obtained (**Appendix Table 0.4**) and after incorrect sequences were discarded (sequences which matched with other than methylacetate reductase enzyme e.g. alcohol dehydrogenase) 20,230 *mar* sequences were analysed and grouped within 13 OTUs picked from T₀ and PNP degrading river water. Analysis of alpha diversity of *mar* sequences revealed a higher diversity of *mar* in PNP degrading river water than in T₀ samples. Difficulties with *mar* amplification from T₀ river water samples resulted in much lower number of sequences from those samples except for replicate three of T₀ river water.

The OTU profiles (OTUs formed by more than 1% of total *mar* sequences per sample) (**Figure 2.13**) were compared. *mar* sequences from T₀ samples formed mainly OTUs 45, 99, 144, and 171, whereas OTUs 0, 1, 8, 82, 104, 134, 135, 159 and 181 consisted of *mar* sequences from HD samples. High variability of replicates was observed at T₀ and after PNP degradation in the dark.

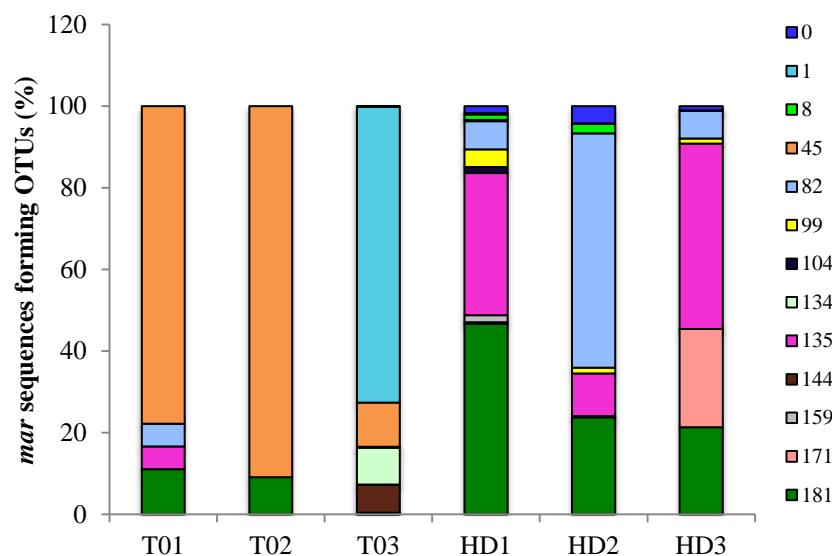


Figure 2.13: The relative abundance of *mar* sequences as % of total *mar* sequences forming OTUs from T₀ and river water degrading high PNP concentration (HD). Where: OTU numbers are given in the figure legend; T₀1-T₀3 –replicates of freshly collected river water, HD1-HD3- replicates of river water degrading high PNP concentration in the dark.

Representative *mar* sequences from picked OTUs were analysed using BLAST (NCBI). OTUs present in dark river water which degraded PNP were OTU number 0, 1, 8, 82, 104, 134, 135, 159 and 181 (**Table 2.10**). Representative *mar* sequences from those OTUs resulted in the closest relation to *mar* sequences from *Azospirillum lipoferum* 4B, *Pseudomonas* sp. GM74, *Cupriavidus taiwanensis* LMG 19424, *Novosphingobium resirovorum*, *Rhizobium leguminosarum* bv. *viciae* 3841, *Pseudomonas* sp. WBC-3, *Pseudomonas fluorescens* F113 and *Sphingobium chlorophenolicum* L-1, respectively. OTUs 45, 99, 144 and 171 were mainly present at T₀ with representative sequences similar to *mar* from *Hoeflea phototrophica* DFL-43, *Pseudomonas fluorescens* F113, *Burkholderia cepacia* and *Burkholderia* sp. SJ98, respectively. Few OTUs (0, 82 and 135) consisted of *mar* sequences from both, T₀ and PNP degrading river water.

It is noteworthy that both *pnpA* and *mar* gene sequences were detected in *Pseudomonas syringae* isolate AKHD2, and several *pnpA* and *mar* sequences derived from clone libraries and 454 sequencing (**Table 2.9** and **2.10**) showed the closest match to *pnpA* and *mar* gene from a known PNP-degrader *Pseudomonas* sp. WBC-3 (Zhang *et al.*, 2009).

Table 2.10: Identity and relative abundance of *mar* gene sequences in picked OTUs from T₀ river water and from river water degrading PNP at high concentration in the dark (HD).

Sample	OTU No.	% total sequences	BLAST result (accession number)	%ID	References
T ₀ + HD	0	21.74	maleylacetate reductase <i>Azospirillum lipoferum</i> 4B (YP_004974335.1)	65	Wisniewski-Dyé <i>et al.</i> , 2011
HD	1	15.40	MalI (maleylacetate reductase) <i>Pseudomonas</i> sp. GM74 (AC053215.2)	90	-
HD	8	0.06	maleylacetate reductase <i>Cupriavidus taiwanensis</i> LMG 19424 (YP_001795808.1)	67	Amadou <i>et al.</i> , 2008
T ₀	45	0.42	putative maleylacetate reductase <i>Hoeflea phototrophica</i> DFL-43 (ZP_02165041.1)	60	-
T ₀ + HD	82	32.96	maleylacetate reductase <i>Novosphingobium resirovorum</i> (AAW29743.1)	70	-
T ₀	99	0.46	maleylacetate reductase <i>Herbaspirillum frisingense</i> (WP_006462094.1)	42	-
HD	104	0.05	maleylacetate reductase <i>Rhizobium leguminosarum</i> bv. <i>viciae</i> 3841 (YP_764617.1)	84	Young <i>et al.</i> , 2006
HD	134	1.30	maleylacetate reductase <i>Rhizobium leguminosarum</i> bv. <i>viciae</i> 3841 (YP_764617.1)	77	Young <i>et al.</i> , 2006
T ₀ + HD	135	20.49	putative maleylacetate reductase <i>Pseudomonas</i> sp. WBC-3 (ABU50914.1)	94	Zhang <i>et al.</i> , 2009
T ₀	144	0.72	putative maleylacetate reductase <i>Burkholderia cepacia</i> (AAL50016.1)	79	Johnson <i>et al.</i> , 2002
HD	159	0.69	protein PnpD (maleylacetate reductase) <i>Pseudomonas fluorescens</i> F113 (YP_005208840.1)	93	Redondo-Nieto <i>et al.</i> , 2012
T ₀	171	3.78	maleylacetate reductase <i>Burkholderia</i> sp. SJ98 (WP_008346113.1)	88	-
HD	181	1.90	maleylacetate reductase <i>Sphingobium chlorophenolicum</i> L-1 (YP_004554683.1)	57	-

Representative sequences for *mar* OTUs were analysed in ARB as described above. Sequences were included in the *mar* tree (**Figure 2.11**). OTUs were grouping within eight major clades together with sequences obtained from clone libraries. Diversity of *mar* sequences once again revealed the presence of α , β , and γ -*Proteobacteria* (**Table 2.10**). While *Hoeflea phototrophica* DFL-43, *Herbaspirillum frisingense*, *Burkholderia cepacia* and *Burkholderia* sp. JS98 seemed to be prevalent in T₀ river water, presence of *Pseudomonas fluorescens* F113, *Pseudomonas* sp. GM74, *Sphingobium chlorophenolicum* L-1, *Rhizobium leguminosarum* bv. *viciae* 3841 and *Cupriavidus taiwanensis* LMG 19424 was observed in HD river water. Moreover, increased abundance of three bacteria present at T₀ *Azospirillum lipoferum* 4B, *Novosphingobium resirovorum* and *Pseudomonas* sp. WBC-3 was noticeable in HD samples.

Taxonomy information from previously described *mar* clone library was used to assign taxonomy to the picked OTUs for *mar* sequences. Results of 454 sequencing were with good agreement with the *mar* clone library (**Figure 2.11**).

2.5. Discussion

2.5.1. Effect of light on the biodegradation of PNP and river water bacterial community structure

The initial hypothesis that light impacts on PNP biodegradation was supported by data obtained in current study. Increased river water pH inhibited the biodegradation of PNP in river water (**Figure 2.4**). The effect of pH (**Table 2.6**) was correlated with the proliferation of algae under light conditions (Section 2.2.1). Light conditions induce the production of algal biomass in the biodegradation test systems due to the carbon fixation in the process of photosynthesis (McKendry, 2002). The assimilation of carbon dioxide from carbonic acid present in water decreases the acidity of water and as a result pH is increased. The inhibition of PNP biodegradation could also be caused by algae suppressing the PNP degrading communities, which resulted in the loss of community biodegradation potential. This could explain the absence of TRF 245 nt (previously characterized for PNP degrading isolate *Pseudomonas syringae* AKHD2) in the TRFLP profile of river water incubated under light (**Table 2.7**).

Additional experimental work with PNP degrading isolates (**Figure 2.5**) in the absence of phototrophs revealed that there was no direct effect of light on the biodegradation of PNP, and that enzymes involved in the biodegradation of PNP were not light sensitive. It was found that pH was the key factor inhibiting PNP biodegradation (**Figure 2.6 a and b**). This was possibly due to changes in PNP uptake in liquid medium with increased pH, and therefore, lack of bacterial biodegradation. In solution PNP has $pK_a=7.16$ at 22 °C and it was reported by Li *et al.*, (2008) that alkaline conditions are more suitable for effective PNP biodegradation. However, decrease in PNP biodegradation rate was observed when pH was higher than 9.0. According to European Commission datasets, pH can affect the PNP hydrolysis and photodegradation. Although, PNP is stable in water at pH 5.0-9.0 ($DT_{50} > 50$ days) PNP photodegradation increases with lower pH and complete photodegradation of PNP in water, at a concentration of 200 mg/L, would occur after 2 months (ESIS, 2012).

Although incubation of river water in light resulted in increased pH which lead to inhibition of PNP biodegradation, some phototrophic PNP-degrading organisms have been characterized by Roldán *et al.* (1998) and Lima *et al.* (2003) and PNP biodegradation under light conditions is possible. Algal biodegradation of crop protection products in light was also shown by Thomas and Hand (2011) in aquatic ecosystems leading to significant increases in the rate of biodegradation of a range of chemicals under light conditions in comparison with the dark. Lin *et al.* (2010) also reported faster biodegradation of fluoroquinolone antibiotics in pond water and sediment under light conditions than the dark. Moreover, slurry experiments conducted by Abed *et al.* (2002) revealed additional groups of light enriched bacteria degrading petroleum compounds which were absent from dark enriched consortia of degrading bacteria. Light may also induce changes in the ratio between algal and microbial populations present in tested inocula, and therefore, impact on the outcome of biodegradation tests. For example, availability of light can modulate the structure and function of biofilms and light enhance the growth of algae and bacteria, and stimulates algal-bacterial coupling in microbial biofilms present in the water ecosystems (Ylla *et al.*, 2009). Although, light is a major issue in the biodegradation test systems the inhibition of the biodegradation of chemicals is less likely to occur under light condition in the natural environment i.e. surface river water due to the low probability of such strong pH changes as observed in the laboratory system. One of the reasons for that could be the constant contact of river water with the atmosphere which enables the exchange of CO₂, and therefore, its replenishment in the short term.

2.5.2. Effect of PNP concentration on the biodegradation of PNP and river water bacterial community structure

The present study revealed the effect of the PNP concentration on biodegradation test results. It was found that for both types of inocula, river water (**Figure 2.4**) and bacterial strains (**Figure 2.5**), the biodegradation of PNP at lower concentration does not occur within the same time frame, if at all, as PNP biodegradation at higher concentration. These findings supported the initial hypothesis that the concentration of the test compound (PNP) has an effect on the outcome of the tests and highlights

the limited relevance of high chemical concentrations used routinely in biodegradation tests according to OECD guidelines which induce growth linked catabolism, whereas low concentrations may not be effectively degraded.

The ANOSIM for bacterial TRFLP profiles showed no significant differences in bacterial communities from river water incubated with low and high PNP concentration. Although, shifts in bacterial population were detected in river water degrading high PNP concentration in the dark in comparison with the lower PNP concentration these are likely to be due to proliferation of PNP degrading bacteria which occurred at high PNP concentration but were absent at lower PNP concentration. This suggests that the initial hypothesis that PNP concentration impacts on the bacterial community structure was incorrect. An effect of chemical concentration on microbial community diversity was reported by Margesin *et al.* (2007). Low concentrations did not affect microbial community structure while high Total Petroleum Hydrocarbon (TPH) concentration increased significantly the abundance of Gram-negative bacteria in soil. The population of Gram-positive bacteria was not influenced by TPH concentration. Langworthy *et al.* (1998) also observed that moderate to high concentrations (up to 217 µg/g dry weight of sediment) of polycyclic aromatic hydrocarbons (PAH) altered river sediment microbial communities. Higher numbers of Gram-negative bacteria together with the increased frequency of PAH degradative genes were observed in the highly contaminated site in comparison with ambient sediments, where PAH concentration ranged from below the detection limit to 1.5 µg/g dry weight of sediment.

The present study revealed analytical difficulties that have been encountered when measuring PNP at low concentration (< 20 µg/L), and therefore, more sensitive methods (¹⁴C PNP approach) were used to study the biodegradation of PNP at low concentrations. The importance of using environmentally realistic concentrations, i.e the application of low levels of chemicals in biodegradation studies was also highlighted. Although, the biodegradation of test compound may occur at higher concentrations those results may not be used to accurately predict the biodegradation of chemical in natural ecosystems, occurring at low concentrations. Chemical concentration will influence the adaptation phase of microbial populations (Spain and van Veld, 1983) and chemical biodegradation kinetics (Toräng *et al.*, 2003)

leading to slower or lack of chemical degradation at low concentrations. Therefore, extrapolation of test results from laboratory conditions to environmental compartments could be also challenging and may lead to incorrect conclusions on chemical persistence and chemical risk assessments.

2.5.3. Identification of PNP degrading bacterial populations

In order to identify PNP-degrading bacterial populations present in river water, culture dependent and independent methods were applied. A culture dependent approach was useful for isolation of PNP degrading bacteria from river water (**Table 2.5**) while culture independent microbial ecology methods i.e. TRFLP for 16S rRNA gene, enabled bacterial community profiling and identification of bacteria which proliferated during biodegradation, and hence were potential degraders (**Figure 2.8**). Increased abundance of TRF 245 nt, identified as *Pseudomonas syringae* AKHD2, indicated proliferation of PNP-degrading bacteria in river water samples (**Table 2.7**). Increased presence of *P. syringae* correlated with increased number of copies of PNP degradation genes *pnpA* and *mar*. It is noteworthy that both PNP functional markers were detected from *P. syringae*. Based on that it can be suggested that *Pseudomonas* and in particular *Pseudomonas syringae* was a major degrader of PNP in this system.

The PNP functional markers analysis enabled detection and identification of additional PNP-degraders. Analysis for *pnpA* and *mar* functional markers (**Figure 2.10**, **2.11** and **Table 2.8**) revealed the diversity of PNP degraders involved in the first and last stage of PNP biodegradation. The initial sequencing results and clone libraries for *pnpA* and *mar* indicated higher diversity of *mar* than *pnpA* and suggested that the *Pseudomonas*-like bacteria play the major role in the biodegradation of PNP. The discrepancy between the diversity of bacteria involved in the first and last step of the PNP biodegradation pathway could be due to the primer design for functional genes *pnpA*, *npdA2* and *mar*. It is noteworthy that *pnpA* and *npdA2* primers were restricted to one genus of Gram-negative and two genera of Gram-positive bacteria, respectively. While *pnpA* primers targeted *Pseudomonas*-like bacteria, amplification of *npdA2* gene was successful from *Arthrobacter* sp.

JS443, *Arthrobacter chlorophenolicus* A6 and *Rhodococcus opacus* B4. However, *mar* primers were designed based on a conserved fragment in *mar* sequences obtained from a range of bacterial strains including for α , β , and γ - *Proteobacteria*. As a result amplification of *mar* was possible from a variety of control bacterial strains (**Table 2.2**) and isolates obtained from experimental samples (**Table 2.5**).

High throughput data analysis (**Table 2.9** and **2.10**) confirmed the results from clone libraries (**Figure 2.10** and **2.11**), and also showed higher diversity of *mar* than *pnpA* functional marker. Additionally, analysis of 454-reads revealed false positive results which arrived from *pnpA* and *mar* sequencing and were present mainly in T₀ river water with higher level of false results for *pnpA* than *mar* gene. This could explain why QPCR for *pnpA* and *mar* indicated higher number of copies of *pnpA* than *mar* in environmental samples (**Figure 2.9 a** and **b**). However, the copy number for *mar* does not represent only PNP-degraders, and the counts for 16S rRNA are very low which might suggest that they do not represent the actual abundance of bacteria in the samples.

It can be speculated that low diversity of *pnpA* and lack of detection of *npdA2* gene was because some PNP-degrading bacteria can be represented by rare species that have a slow growth rate and are in relatively low concentration of biomass. Higher diversity of *mar* could suggest the presence of chemical-degrading bacteria which share the same last reaction with PNP biodegradation pathway, which is catalysed by maleylacetate reductase, before metabolites derived from chemical biodegradation enter the TCA cycle. It is well documented that *mar* could be involved in the biodegradation of many aromatic chemicals (Seibert *et al.*, 1993). Overall, the *pnpA* functional marker was found to be a more accurate tool to study the diversity of PNP-degrading bacteria since *mar* primers were targeting much broader group of bacteria degrading other than PNP nitroaromatic compounds. Further experimental work would be essential to answer the question regarding usefulness of application of *mar* functional marker in OECD biodegradation tests with different chemical compounds.

2.5.4. PNP biodegradation pathway

The results suggested the predominance of a specific PNP biodegradation pathway in the experimental systems. According to Chauhan *et al.* (2010) the biodegradation of PNP may occur through two major catabolic pathways. The first one was characterized for Gram-negative bacteria and involves gene *pnpA* encoding the 4-PNP monooxygenase enzyme which catalyses the first step in the PNP biodegradation (Zhang *et al.*, 2009). The second pathway was well described for Gram-positive bacteria and the first reaction is catalysed by PNP 2-monooxygenase encoded by gene *npdA2* (Perry and Zylstra, 2007; Chauhan *et al.*, 2010). Detection of *pnpA* but absence of *npdA2* gene in river water before and after PNP biodegradation might suggest that the biodegradation of PNP followed the pathway characterized for Gram-negative bacteria. Moreover, proliferation of *Pseudomonas* during the biodegradation of PNP indicated that Gram-negative bacteria were the main degraders and that the Gram-negative pathway played a key role in the biodegradation of PNP. The last enzyme involved in the biodegradation of PNP is the maleylacetate reductase encoded by *mar* (Seibert *et al.*, 1998; Chauhan *et al.*, 2010). The presence of *mar* in both isolate *P. syringae* AKHD2 and environmental samples confirmed that the last stage of PNP biodegradation pathway was also involved in the PNP biodegradation with river water incubated under dark.

2.6. Conclusions

Based on results presented in current study it was concluded that:

1. Increased pH inhibited the biodegradation of PNP, since changes in pH of river water alter the chemical speciation of PNP and associated ion, and may thus affect the uptake of PNP into PNP-degrading bacteria. This is an artefact of the test system which would not occur in the river under normal circumstances except in eutrophic conditions.
2. PNP test concentration affected biodegradation kinetics and biodegradation was observed only at a high PNP concentration with both, bacterial isolates and environmental samples.
3. Even at the high PNP concentrations used, PNP did not impact the microbial community structure. The biodegradation of PNP at high concentration by river water microbial populations indicated that PNP is not toxic to bacterial communities.
4. Introducing environmental complexity to biodegradation tests may lead to unpredictable effects on PNP biodegradation in experimental systems.
5. The biodegradation of PNP occurred via the pathway characterized for Gram-negative bacteria.
6. *Pseudomonas syringae* was the key PNP-degrader present in river water samples.
7. Poor quality of QPCR was observed due to non-target hits present in copy numbers for PNP functional markers and very low copy number for 16S rRNA gene.
8. Functional marker *pnpA* was found to be useful in detection and identification of uncultivable groups of PNP-degrading bacteria.
9. *mar* functional marker could be applied for identification of bacteria involved in biodegradation of chemicals when genes involved in the first step of their biodegradation are not characterised without need to develop specific probes.

Chapter 3

The influence of microbial inoculum and water chemistry on the biodegradation of *para*-nitrophenol

3.1. Introduction

In anthropogenic environments, the discharge of water from wastewater treatment plants (WWTPs) has a major impact on the health of aquatic ecosystems and may cause local or global disturbances of community structure and function (Féray and Montuelle, 2003; Wakelin *et al.*, 2008). WWTPs effluent discharge can introduce large amounts of organic matter, nutrients and man-made chemicals including personal care ingredients, pharmaceutical compounds and hormones into receiving waterways (Akiyama and Savin, 2010; Wakelin *et al.*, 2008; Batt *et al.*, 2006; Ekka *et al.*, 2006; Rodgers-Gray *et al.*, 2000). Large inputs of nitrogen and phosphorus (Servais *et al.*, 1999; Nhapi and Tirivarombo, 2004) can lead to eutrophication and temporary oxygen deficits due to the limitations of stream ecosystems in removing these nutrients (Marti *et al.*, 2004). Effluent discharge can also deposit sand and grit into aquatic systems, affecting the physical characteristics of sediment. The discharge itself can perturb the natural flow regime, particularly when it enters waterways during periods of low natural flow leading to changes in water quality, fish and microbial communities and bacteria-mediated processes (Féray and Montuelle, 2003). Effluent discharged from WWTP may also introduce some active bacterial species from WWTP into the streams, and therefore, increase their abundance in receiving waters (Goñi-Urriza *et al.*, 1999) which can be beneficial for biodegradation processes occurring in rivers.

Microbial populations present in natural ecosystems play a key role in the so-called self-purification processes of aquatic systems. Bacteria and other microorganisms are ubiquitous and highly abundant in river sediments, biofilms and the water column, and are good indicators of the condition of aquatic ecosystems (Lawrence *et al.*, 2005). Moreover, bacteria are responsible for biogeochemical transformations, such

as nitrification and denitrification (García-Ruiz *et al.*, 1998) and the function of bacterial communities is strictly related to their composition (Carlson *et al.*, 2004; Johnson *et al.*, 2004; Kirchman *et al.*, 2004). Stress and disturbance of microbial communities may impact on ecosystem function and processes as well as biodiversity and aquatic community structure (Wakelin *et al.*, 2008). According to Chénier *et al.* (2003) the addition of nutrients to river water shifted river biofilm community structure from one dominated by bacterial biomass to biofilms dominated by algae. Wakelin *et al.* (2008) reported a strong cause-and-effect relationship between the loading of carbon and nitrogen into the stream from the WWTP outfall and responses in bacterial abundance, community structure, and function, showing an extensive influence of the WWTP on the ecology of the stream sediment.

A variety of man-made chemicals are present in anthropogenically influenced environments including pesticides (Claver *et al.*, 2006), pharmaceuticals (Daughton and Ternes, 1999) and compounds used in production of HPCs (Eriksson *et al.*, 2003; Ying, 2006). Such chemicals affect ecosystems mainly due to chemical persistence and toxicity to plants, fish and microbial communities (Kümmerer *et al.*, 2004), and hence, lead to the disturbance of processes conducted by those organisms. Biodegradation of chemicals is a crucial process in the self-purification of contaminated environments. Differences in bacterial community structure may affect the community function in the environment. For instance, a study by Näslund *et al.* (2008) indicated that antibiotic pollution can affect bacterial diversity and associated biodegradation potential. Polyaromatic hydrocarbon degrading bacteria in marine sediments were affected by ciprofloxacin present in the effluent outfall of a WWTP and inhibition of pyrene mineralization was observed with increasing ciprofloxacin concentration. Bradley *et al.* (2009) assessed the potential for microbial biodegradation of reproductive hormones in effluent-affected streams. It was found that mineralization of estrone (E1) and testosterone (T) was affected in sediment downstream of WWTP outfalls, in comparison with upstream sediment while 17 β -estradiol (E2) mineralization was faster in downstream sediments. Chénier *et al.* (2003) found that bacterial production was limited by the availability of nutrients in the river water and that nutrient amended biofilms had higher potential to mineralise hexadecane. In order to determine chemical persistence in the environment, the

Chemical Industry typically investigates compound biodegradability using standardised OECD tests. Samples are collected from different environmental compartments and used as inocula in OECD tests, which vary depending on the type of tests. There is no prescribed inoculum standardization in OECD tests guidelines, which is a potential source of error influencing test results. Reference materials are known as important tools for maintaining high-quality assurance standards, including for biological materials e.g. inocula (Paixão *et al.*, 2006). Inoculum size, origin and especially the presence of microbial populations capable of biodegradation of chemicals should be considered in biodegradation studies. Only a few studies have assessed the effect of inoculum and the number of specific degraders on the outcome of biodegradation tests (Thouand *et al.*, 1995; Ramadan *et al.*, 1990; Ingerslev *et al.*, 2000). It remains to be established how the extent of in-stream biodegradation of chemicals may correlate with variation in the communities of specific degraders present in river water or sediment and how these communities may vary spatially in aquatic ecosystems.

Mezzanotte *et al.* (2005) showed that different biodegradation activities could be found for activated sludge from two municipal WWTPs, and therefore, low reproducibility of OECD tests can be caused by the variability of the inoculum i.e. samples of activated sludge withdrawn from different WWTPs. Comber and Holt (2010) indicated that false negative results in biodegradation tests could be associated with low levels of biomass in the inoculum and absence of competent degraders. The effect of inoculum source on biodegradation potential was reported by Moreno and Buitrón (2004). Inocula sampled from aeration tanks from domestic, municipal and industrial WWTP revealed different specific activities for 4-chlorophenol biodegradation. Due to the variability of test inocula the mechanisms responsible for biodegradation of chemicals may also be different. Uncertainty of mechanisms may lead to misinterpretation of test results and incorrect prediction of the fate of chemicals under environmentally relevant conditions in ecosystems with different physicochemical conditions and community structures.

Inoculum variability can result from the existence of a variety of microhabitats within the same environment, as well as differences in nutrient requirements and their availability in different microhabitats (Swindoll *et al.*, 1988). Inoculum

composition especially in terms of the abundance of rare species, which may carry functional genes essential for chemical biodegradation (Szabó *et al.*, 2007), is important for determining biodegradation rate of chemicals. According to Thouand *et al.* (2011) the presence and density of rare groups of species in the inoculum is influenced by the history and origin of that sample. It was also suggested that specific degraders for non-readily-degradable substances are more likely to be found in the sediment than the water of rivers and coastal areas, whereas samples from pristine areas not only have low total cell numbers but may also contain very small numbers of specific degraders. Therefore, the choice of sampling site for inoculum collection may affect the inoculum composition and its potential for biodegradation of chemicals, which may lead to false positives or negatives in biodegradation tests, and/or inconsistent test results making the use of biodegradation studies to inform risk assessment and chemical management uncertain.

3.2. Objectives

The objective of the experiments described here was to determine the relationship between inoculum source, size and chemical biodegradation. The following hypotheses were tested: (1) the biodegradation rate of *para*-nitrophenol (PNP) is not affected by the inoculum type; (2) WWTP effluent does not affect the potential of the inoculum to degrade PNP; (3) microbial community composition is not affected by sampling site; and (4) WWTP effluent does not affect the bacterial community composition downstream of the effluent outfall.

3.3. Materials and methods

3.3.1. Sample collection and preparation

In November 2010, surface river water and sediment were collected 530 m downstream (**Figure 3.1** location **D** and **E**) and 200 m upstream (**Figure 3.1** location **A** and **B**) of the Wellesbourne WWTP effluent discharge point (**Figure 3.1** location **C**). Additionally, river water from the effluent outfall was also collected. The river water and sediment samples were used as inocula in a PNP biodegradation experiment. The surface water collected had a temperature of 16-17 °C. In order to remove particles and large protozoa, water was filtered through a 38 µm sieve. River water for the controls was sterilized in three autoclaving cycles (121 °C, for 15 minutes, 1.1 atm). Both, upstream and downstream sediments were mixed sediments and consisted of sand, silt and gravel, with the majority of particles 2-3 mm. Sediment was used to prepare sediment extracts according to Vázquez-Rodríguez *et al.* (2008). The top 5 cm layer of sediment was removed and 13 g of wet sediment (60-70% water content) was suspended in amber bottles with 400 mL of upstream or downstream sterile river water. Bottles were shaken on the rotary shaker for 30 minutes at 20±2 °C and agitation at 100 rpm. After settling for 30 minutes, 300 mL of supernatant was decanted and used as inoculum. Biodegradation tests were carried out based on OECD 309 simulation tests for aerobic chemical biodegradation in surface water (OECD Guidelines, 2002).

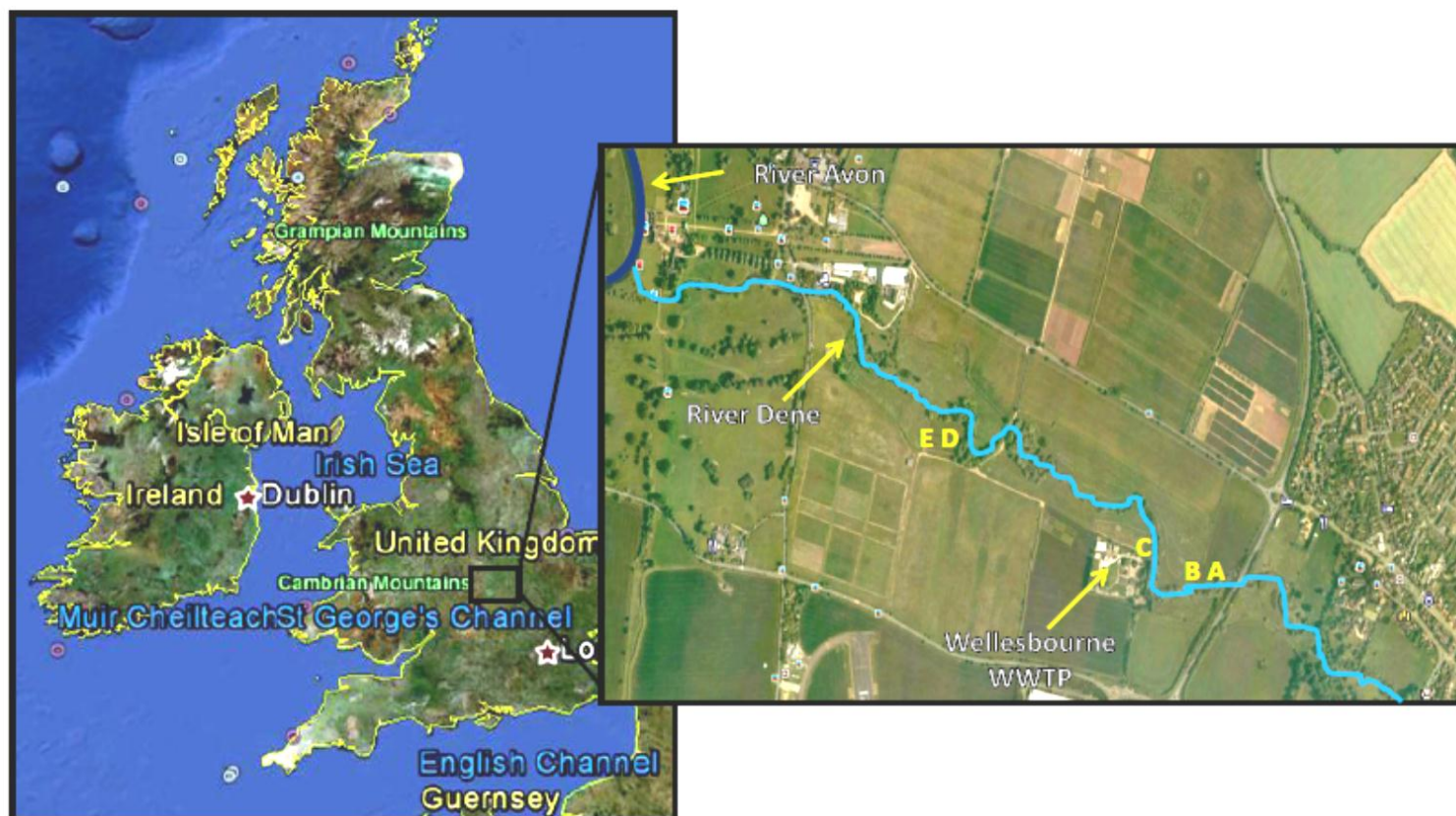


Figure 3.1: River Dene and location of sampling points. Collection point for: **A**- river water upstream of WWTP; **B**- sediment upstream of WWTP; **C**- river water with effluent from WWTP; **D**- river water downstream of WWTP and **E**- sediment downstream of WWTP (source: Google Earth, 2012).

3.3.2. Experimental set up

Sterile Duran Schott amber bottles (500 mL) (Fisher Scientific, UK) were filled with river water or sediment extract as an inoculum (300 mL). Treatments were prepared in triplicate. PNP, Reagent Plus® ≥99% (Sigma–Aldrich, UK) was used to prepare an aqueous solution containing 1200 mg/L of substrate which was used for spiking water and sediment extracts to an initial final concentration of 2 mg/L PNP. The ratio between the total capacity of the bottle (500 mL) and the volume of river water or sediment extract (300 mL) matched that of Toräng and Nyholm, (2005). For the positive control, sterile river water with PNP was inoculated with induced PNP-degrading isolate *Pseudomonas syringae* AKHD2 grown on LB medium with PNP (56 mg/L) for 48 hours at 25 °C. A negative control consisted of sterile river water. The experiment was run in a controlled environment room in the dark. Temperature was 20±2 °C, and bottles were incubated under constant motion using a rotary shaker (MK V Shaker, L.A. Engineering Co., UK) at 50 rpm. PNP concentration was measured at regular intervals. Inoculum size was assessed at T₀. After 100% biodegradation of PNP determination of number of PNP degraders and DNA extraction from medium was performed.

3.3.3. Measurement of PNP biodegradation

PNP was analysed by HPLC as described in Chapter 2, section 2.3.3.

3.3.4. Measurement of ammonium, nitrite and nitrate

Determination of ammonia and the sum of nitrite/nitrate was performed using a FIAstar 5000 flow injection analyser (FOSS Ltd, UK). To determine the ammonium concentration water samples and sediment extracts (20 mL) were injected into a carrier stream which merged with a sodium hydroxide (NaOH) stream. In the resulting alkaline stream gaseous ammonia is formed and diffused through into an indicator stream with a mixture of acid-base indicators (boric and sulphuric acid). A

colour shift results which can be measured photometrically at 590 nm. For the quantification of the sum of nitrite/nitrate, river water samples and sediment extracts were mixed with phosphate buffer. Nitrate was reduced to nitrite in a cadmium reactor. On the addition of a sulphanilamide solution, nitrite initially present and nitrite formed from reduction of nitrate formed a diazo compound. This compound was coupled with N-(1-naphtyl)-ethylene diamine dihydrochloride (NED) to form a purple azo dye. Absorbance was measured at a wavelength of 540 nm.

3.3.5. Determination of total viable count (colony forming units)

3.3.5.1. Inoculum size

As described in Chapter 2, section 2.3.5.1.

3.3.5.2. Isolation of specific degraders and phylogenetic tree for 16S rRNA gene

Isolation and identification of PNP-degraders was performed as described in Chapter 2, section 2.3.5.2. RDP classifier (Wang *et al.*, 2007) was used to assign 16S rRNA gene sequences obtained from isolates. A neighbour joining tree with 16S rRNA gene sequences was created using ARB software version 5.3-private-210 (Ludwig *et al.*, 2004). 16S rRNA gene sequences from PNP-degrading isolates were imported into ARB and pre-alignment was created with 16S rRNA gene sequence from *Pseudomonas syringae* AB167245 from Silva database version 106. Sequence alignment was corrected according to secondary structure criteria using ARB_EDIT4. Pristine Silva database was used to select reference species which were included in the tree. A neighbour-joining tree was created based on aligned nucleotide sequences and their distance matrix using jukes-cantor correction available in ARB (Ludwig *et al.*, 2004). The tree was built based on the conserved nucleotide sequence (*E.coli* position 102-988, 887 valid columns) and 16S rRNA gene sequence from *Pseudomonas fluorescens* Pf0-1 as a filter. 16S rRNA gene sequence from isolate 1 U2 was excluded from the tree due to the short nucleotide sequence. Bootstrap analysis (100 replicates) was performed using ARB software (Ludwig *et al.*, 2004).

3.3.6. Colony PCR

As described in Chapter 2, section 2.3.6.

3.3.7. Terminal Restriction Fragment Length Polymorphism (TRFLP)

As described in Chapter 2, section 2.3.9. Replicates of each group of TRFLP profiles (river water, sediment extract, effluent, upstream site, downstream site) from each time point (T_0 and T_{end}) were pooled together and average TRFLP profiles were used to perform additional 2D bubble plots in Primer 6. Variation between TRFLP profiles was determined based on Index of Multivariate Dispersion (IMD) (Warwick and Clarke, 1993) which was derived using option MVDISP available in Primer 6 which compares multivariate dispersion across treatments.

3.3.8. Detection of PNP functional genes

As described in Chapter 2, section 2.3.12.

3.3.9. Quantitative PCR of *pnpA* and *mar* genes

As described in Chapter 2, section 2.3.15.

3.3.10. Statistical analyses

As described in Chapter 2, section 2.3.18. ANOVA for inoculum size and number of PNP-degrading bacteria was calculated based on log transformed values using GenStat. Correlation between the inoculum size and the number of PNP degrading bacteria detected in the treatments, as well as correlation between the number of PNP-degraders, lag phase, PNP degradation rate and DT values were determined using function CORREL in Microsoft Excel. Correlation coefficients were compared with Critical Values of the Pearson Product-Moment Correlation Coefficient table to determine whether there is a statistically significant relationship between compared factors at $P < 0.05$.

3.4. Results

3.4.1. The biodegradation of PNP by different inocula

PNP was degraded within eight days for all treatments (**Figure 3.2**). For most environmental samples there was a lag phase of between 3-5 days before rapid PNP biodegradation occurred. Two of the triplicates of upstream sediment (site **B**) and one of the triplicates of upstream river water (site **A**) had degraded 25-30% after day one, then PNP biodegradation stopped and resumed after three days and was finished between days 5-6. The values for DT_{15} , DT_{50} , DT_{90} , lag phase and maximum PNP biodegradation rate were calculated for all treatments (**Table 3.1**). Significant differences were found between the treatments for lag phase and significantly shorter lag phase was observed in sediment upstream than in river water collected downstream ($P=0.037$). The analysis for DT_{15} values revealed that PNP biodegradation was significantly different in downstream river water compared to upstream river water and sediment extract ($P=0.041$). There were no significant differences between the remaining samples. ANOVA for degradation rate, DT_{50} and DT_{90} values showed no significant difference between treatments ($P=0.612$, $P=0.221$ and $P=0.360$, respectively). No significant differences were found in treatments for the length of the lag phase ($P=0.076$) or maximum PNP degradation rate ($P=0.234$) (**Table 3.1**). Given the high variability of replicates of treatments it was difficult to determine if there are differences in the biodegradation of PNP with different inocula.

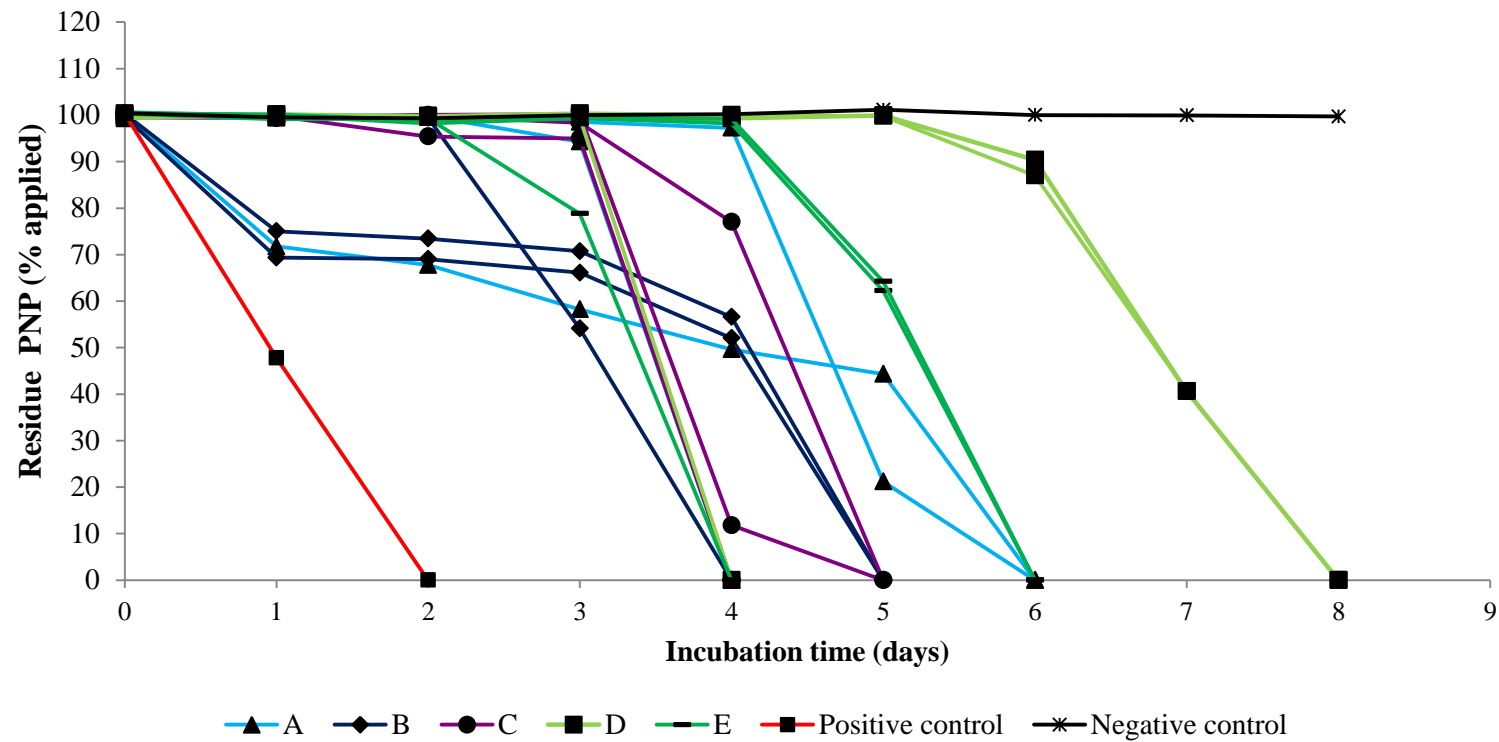


Figure 3.2: The biodegradation of PNP with different inocula.

Figure legend: A- water upstream, B- sediment upstream, C- water at effluent outfall, D- water downstream, E- sediment downstream.

Table 3.1: Statistical analysis for PNP biodegradation in different treatments.
Average values for lag phase, maximum PNP degradation rate and DT values at 15, 50 and 90% degradation of PNP (standard deviation in brackets)

Site	Lag phase (days)	Degradation rate after acclimation (days)	DT ₁₅ (days)	DT ₅₀ (days)	DT ₉₀ (days)
Water upstream	1.83 (±0.93) ^{ab}	3.67 (±1.20) ^a	2.60 (±1.08) ^a	4.02 (±0.33) ^a	5.07 (±0.59) ^a
Sediment upstream	0.67 (±0.67) ^b	4.00 (±1.00) ^a	1.14 (±0.59) ^a	3.74 (±0.33) ^a	4.48 (±0.33) ^a
Effluent	2.43 (±0.57) ^{ab}	2.33 (±0.33) ^a	3.30 (±0.16) ^{ab}	3.80 (±0.28) ^a	4.31 (±0.29) ^a
Water downstream	4.33 (±0.67) ^a	3.00 (±1.00) ^a	5.10 (±0.98) ^b	5.70 (±1.10) ^a	6.47 (±1.29) ^a
Sediment downstream	3.33 (±0.67) ^{ab}	2.33 (±0.33) ^a	3.40 (±0.51) ^{ab}	4.73 (±0.69) ^a	5.75 (±1.06) ^a

Where: treatments with different letters are significantly different ($P < 0.05$); \pm standard error of the mean (S.E.M.), $n=3$.

3.4.2. Water and sediment chemistry

The mean pH and concentration of ammonia, nitrite and nitrate (**Table 3.2**) were determined for river water and sediment extracts before and after PNP biodegradation. Significant differences in pH were found for T_0 samples of sediment extracts in comparison with water samples and downstream river water and sediment had higher pH than effluent and upstream river water and sediment. Levels of ammonia and nitrate were significantly higher in T_0 sediment extracts than in river

water but similar between upstream and downstream sediment. After PNP biodegradation, concentration of nitrite and nitrate were significantly different between effluent and upstream and downstream sediment and river water. Higher levels of nitrite were observed in all treatments after PNP biodegradation potentially due to the oxidative release of NO_2^- from PNP.

Table 3.2: Chemistry of river water and sediment extracts.

Site	pH		$\text{NH}_4\text{-N}$ (mg/L)		$\text{NO}_2\text{-N}$ (mg/L)		$\text{NO}_3\text{-N}$ (mg/L)	
	T_0	T_{end}	T_0	T_{end}	T_0	T_{end}	T_0	T_{end}
Water upstream	7.73 ^a (±0.01)	7.93 ^a (±0.06)	0.09 ^a (±0.00)	0.09 ^a (±0.01)	0.03 ^a (±0.01)	0.16 ^a (±0.03)	11.48 ^{ab} (±0.05)	6.62 ^a (±0.05)
Sediment upstream	7.78 ^b (±0.02)	7.78 ^a (±0.07)	0.15 ^b (±0.02)	0.11 ^a (±0.02)	0.04 ^a (±0.00)	0.05 ^b (±0.02)	7.00 ^c (±0.29)	6.95 ^a (±0.29)
Effluent	7.71 ^a (±0.01)	7.81 ^a (±0.10)	0.08 ^a (±0.01)	0.1 ^a (±0.02)	0.01 ^b (±0.00)	0.22 ^c (±0.01)	14.46 ^b (±0.08)	14.87 ^b (±0.08)
Water downstream	8.04 ^c (±0.02)	7.99 ^a (±0.05)	0.08 ^a (±0.01)	0.08 ^a (±0.01)	0.03 ^a (±0.00)	0.18 ^{ac} (±0.01)	7.67 ^c (±0.05)	8.24 ^c (±0.05)
Sediment downstream	8.18 ^d (±0.03)	7.78 ^a (±0.05)	0.16 ^b (±0.03)	0.09 ^a (±0.01)	0.03 ^a (±0.00)	0.08 ^b (±0.00)	8.73 ^{ac} (±0.12)	8.35 ^c (±0.12)

Where: treatments with different letters are significantly different ($P < 0.05$); ± standard error of the mean (S.E.M.); n=3.

3.4.3. Inoculum size and number of PNP degrading bacteria

Number of cultivable bacterial cells (**Table 3.3**) revealed that the inoculum size varied from 1.03×10^5 cfu/mL in river water at effluent outfall to 8.59×10^6 cfu/mL in upstream sediment extract. ANOVA showed no significant differences between treatments ($P=0.121$).

After PNP biodegradation the number of cultivable PNP-degrading bacteria (**Table 3.3**) varied between treatments and ranged from 6.43×10^4 cfu/mL in river water downstream to 3.15×10^5 cfu/mL in river water upstream. However, one-way ANOVA revealed that there was no significant difference in the number of PNP degraders between treatments ($P=0.164$). Also no statistically significant correlation was found between the initial size of inoculum and the number of PNP degraders detected after PNP biodegradation in experimental samples. Additionally, there was no statistically significant correlation between DT_{15} , DT_{50} and DT_{90} values and the inoculum size (the cultivable number of bacteria in T_0 samples). The number of PNP degrading bacteria detected in the treatments did not correlate with calculated lag phase, PNP degradation rate or DT values. However, higher percentage of PNP degraders was present in effluent samples in comparison with other treatments, and river water had higher percentage of PNP degraders than the sediment treatments.

Table 3.3: Number of cultivable bacteria and average number of PNP degraders in environmental samples.

Site	Number of cultivable bacteria (T_0) (cfu/mL)	Number of PNP degrading bacteria (after PNP biodegradation) (cfu/mL)	Normalized number of PNP degrading bacteria (as % of cultivable inoculum)
Water upstream	1.35×10^6 ($\pm 1.85 \times 10^5$)	3.15×10^5 ($\pm 1.61 \times 10^5$)	23.33
Sediment upstream	4.84×10^6 ($\pm 3.75 \times 10^6$)	8.47×10^4 ($\pm 3.89 \times 10^4$)	1.75
Effluent	5.37×10^5 ($\pm 4.33 \times 10^5$)	2.25×10^5 ($\pm 9.01 \times 10^4$)	41.90
Water downstream	2.54×10^5 ($\pm 3.50 \times 10^3$)	6.43×10^4 ($\pm 1.59 \times 10^4$)	25.31
Sediment downstream	3.88×10^6 ($\pm 1.35 \times 10^5$)	2.93×10^5 ($\pm 3.22 \times 10^4$)	7.55

Where: \pm standard error of the mean (S.E.M.); n=2 for the number of cultivable bacteria, n=3 for the number of PNP degrading bacteria.

3.4.3.1. Bacterial isolates degrading PNP

BLAST analysis of 16S rRNA gene sequences obtained from single colonies (colony PCR) from pure cultures enabled identification of PNP degraders. Bacterial strains degrading PNP, isolated from different environmental samples after complete PNP biodegradation resulted in the closest match to *Pseudomonas putida* W619, *Pseudomonas putida* GB-1, *Pseudomonas syringae* pv. *aesculi* str. 0893_23, *Pseudomonas fluorescens* Pf0-1 and *Pseudomonas entomophila* L48 (**Figure 3.3** and **Table 3.4**). Similar bacterial strains were found upstream, at the effluent site, downstream of the effluent outfall, and in river water and sediment from each sampling point. For example, the isolate with similarity to *P. putida* W619 was detected in both river water and sediment from different sampling points. Also, a strain closely related to *P. fluorescens* Pf0-1 was found in upstream and downstream river water and sediment but not in the effluent. On the contrary, strains related to *P. syringae* pv. *aesculi* str. 0893_23 were isolated from upstream and downstream river water but were not detected in river sediment and effluent. One isolate, related to *P. entomophila* L48, was isolated only from downstream sediment, and it was not detected in the remaining treatments. *Azotobacter* was selected as outgroup (**Figure 3.3**). All isolates were closely related since none of the branches were supported with bootstrap values higher than 29 %. Additionally, RDP classifier (Wang *et al.*, 2007) was used to assign 16S rRNA gene sequences. All sequences were classified to genus *Pseudomonas* with 100% confidence.



Figure 3.3: A neighbour-joining phylogenetic tree of 16S rRNA gene from PNP degrading isolates. Tree was created based on nucleotide sequences of 16S rRNA gene using ARB (Ludwig et al., 2004). Figure legend: **in bold** – 16S rRNA gene sequences from PNP-degrading isolates with number of bacterial isolate and isolation source, where: **U**- upstream river water, **E**- effluent river water, **D**- downstream river water, **SU**- upstream sediment extract, **SD**- downstream sediment extract; **normal** - reference 16S rRNA gene sequences obtained from Silva database version 106; **in blue** – reference 16S rRNA gene sequences which resulted in the closest match with 16S rRNA gene sequences from isolates. Species names are followed by accession numbers. 16S rRNA gene sequence from isolate 1 U2 was excluded from the tree due to the short nucleotide sequence. The scale bar represents 1% sequence divergence. Branches with no values have less than 50% bootstrap support (none of the branches were supported with bootstrap values higher than 29 %).

Table 3.4: PNP degrading bacterial isolates from river water and sediment extracts after complete PNP biodegradation.

No.	ISOLATE	RDP CLASSIFIER RESULT	%ID	Accession No.
1	U2	<i>Pseudomonas putida</i> W619	97	CP000949
2	U2	<i>Pseudomonas putida</i> W619	97	CP000949
3	U3	<i>Pseudomonas putida</i> W619	98	CP000949
4	D2	<i>Pseudomonas fluorescens</i> Pf0-1	98	CP000094
5	E2	<i>Pseudomonas putida</i> W619	98	CP000949
6	E2	<i>Pseudomonas putida</i> W619	98	CP000949
7	E1	<i>Pseudomonas putida</i> W619	98	CP000949
8	E2	<i>Pseudomonas putida</i> GB-1	98	CP000926
9	E3	<i>Pseudomonas putida</i> W619	98	CP000949
10	SU2	<i>Pseudomonas putida</i> GB-1	100	CP000926
11	SU3	<i>Pseudomonas fluorescens</i> Pf0-1	97	CP000094
12	SD1	<i>Pseudomonas putida</i> GB-1	98	CP000926
13	SD1	<i>Pseudomonas putida</i> W619	99	CP000949
14	SD1	<i>Pseudomonas putida</i> GB-1	99	CP000926
15	SD1	<i>Pseudomonas entomophila</i> L48	98	CT573326
16	SD3	<i>Pseudomonas putida</i> W619	98	CP000949
17	SD2	<i>Pseudomonas fluorescens</i> Pf0-1	98	CP000094
18	D1	<i>Pseudomonas syringae</i> pv. <i>aesculi</i> str. 0893_23	99	AEAD01000502.1
19	D3	<i>Pseudomonas syringae</i> pv. <i>aesculi</i> str. 0893_23	97	AEAD01000502.1
20	U2	<i>Pseudomonas fluorescens</i> Pf0-1	98	CP000094
21	SU3	<i>Pseudomonas putida</i> W619	98	CP000949
22	U3	<i>Pseudomonas syringae</i> pv. <i>aesculi</i> str. 0893_23	98	AEAD01000502.1
23	SD1	<i>Pseudomonas putida</i> W619	97	CP000949
24	SD1	<i>Pseudomonas putida</i> GB-1	99	CP000926

Where: **U**- upstream river water, **E**- effluent river water, **D**- downstream river water, **SU**- upstream sediment extract, **SD**- downstream sediment extract.

3.4.4. TRFLP profiling of bacterial communities from environmental samples before and after PNP biodegradation

ANOSIM of TRFLP profiles (**Figure 3.4**) revealed that the bacterial communities of the inocula (T_0) were significantly different to each other, and sediment TRFLP profiles were different from those found for bacterial communities in river water. While, communities in river water upstream and downstream of the effluent outfall did not differ, these samples had different bacterial community structure compared to the sediment extracts from the same sites. No difference was found between bacterial profiles from upstream and downstream sediment extracts. Overall, bacterial communities from river water samples had 40% similarity but differed from bacterial communities from the sediment extracts (only 20% similarity).

Variation in bacterial communities was compared using the Index of Multivariate Dispersion (IMD) (**Appendix Table 0.5**). Higher dispersion was observed at T_0 for effluent TRFLP profiles in comparison to upstream river water (IMD=1) and sediment extracts collected upstream and downstream of effluent discharge point (IMD=0.778), while little difference was observed between effluent and river water downstream (IMD=-0.111). After PNP biodegradation (T_{end}) high variability of bacterial communities from effluent and river water downstream (IMD=1) was observed, and the most negative IMD=-1 was found when comparing effluent and upstream river water TRFLP profiles. Therefore, sampling site location affected the variability of bacterial communities, but the increased dispersion did not correlate with increasing distance between sampling sites. Comparison was also performed between T_0 and T_{end} TRFLP profiles. Higher dispersion (IMD=0.778 and 1) was noticeable between bacterial TRFLP profiles from T_{end} river water downstream and profiles of T_0 river water upstream, sediment upstream and sediment downstream and the maximum IMD (IMD=1) was obtained when comparing TRFLP profiles from effluent at T_0 and T_{end} . Highly negative values (IMD= -1 and -0.778) were found in comparison between TRFLP profiles from T_{end} river water upstream and profiles of T_0 river water upstream, sediment upstream and sediment downstream. Overall, it can be suggested that changes in variability of microbial communities are affected by inoculum material, sampling site location and PNP biodegradation.

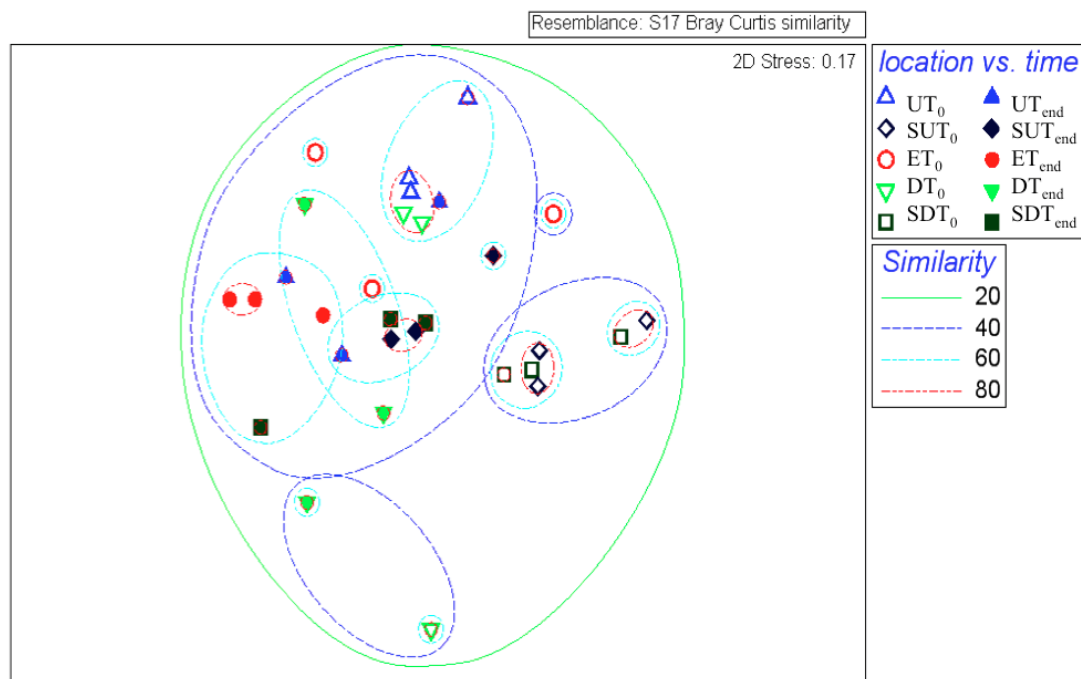


Figure 3.4: Non-metric multidimensional scaling (NMDS) plot of bacterial TRFLP profiles, using Bray-Curtis similarity for the data set derived from amplification with bacterial primers 63F and 1087R- VIC fluorescently labelled and digested with *MspI* and *HhaI*. Stress: 0.17. Figure legend: bacterial TRFLP profiles from U-river water upstream, SU-sediment extract upstream, E-effluent river water, D-river water downstream, SD-sediment extract downstream, T₀- freshly collected river water and sediment extracts, T_{end}- river water and sediment extracts after biodegradation of PNP.

ANOVA for total number of TRFs present in all TRFLP profiles (**Table 3.5**) revealed no differences between the location and inoculum material. No differences were found between effluent and other sampling sites. Additionally, no increase in the number of TRFs was observed in TRFLP profiles from river water and sediment extracts after PNP biodegradation.

Additional analysis of bacterial TRFLP profiles with Primer 6 using 2D bubble plots revealed the proliferation of PNP-degrading bacteria as TRF 245 nt (identified previously as *Pseudomonas syringae*), which was observed after PNP degradation in all treatments (**Figure 3.5 a**). At T_0 TRF 245 nt was significantly more abundant (**Table 3.6**) in effluent than in river water and sediment extracts upstream and downstream of the effluent outfall (**Figure 3.5 a**). Similar abundance of the TRF 245 nt was observed in upstream and downstream river water and sediment extracts after PNP biodegradation. The relative abundance of TRF 245 nt increased in TRFLP profiles after PNP biodegradation in comparison with T_0 profiles. In terms of the presence of TRF 245 nt in inocula, there was no significant difference in its abundance between river water and sediment extracts (**Figure 3.5 b; Table 3.6**). Location affected the presence of TRF 245 nt since it was more abundant downstream of the effluent outfall than upstream of that point (**Figure 3.5 c, Table 3.6**). Overall, no correlation was found between the abundance of TRF 245 nt in inocula TRFLP profiles and their potential to degrade PNP.

Table 3.5: ANOVA for average number of TRFs present in bacterial TRFLP profiles.

Variable	TRFLP profile	Total number of TRFs	ANOVA		
			P-value		
Treatment	upstream water T ₀	14.0 (± 2.54)	0.907	0.311	
	effluent water T ₀	12.0 (± 2.31)			
	downstream water T ₀	13.3 (± 1.67)			
	upstream sediment T ₀	13.3 (± 2.19)			
	downstream sediment T ₀	15.0 (± 2.31)			
	upstream water T _{end}	16.7 (± 1.44)	0.455		
	effluent water T _{end}	17.3 (± 0.35)			
	downstream water T _{end}	15.7 (± 1.44)			
	upstream sediment T _{end}	18.3 (± 1.21)			
	downstream sediment T _{end}	15.7 (± 0.87)			
Material	water T ₀	13.7 (± 1.91)	0.153	0.204	
	sediment T ₀	14.2 (± 2.08)			
	water T _{end}	16.2 (± 1.32)	0.536		
	sediment T _{end}	17.0 (± 1.27)			
Location	upstream T ₀	13.7 (± 2.14)	0.808	0.175	
	downstream T ₀	13.2 (± 1.91)			
	upstream T _{end}	17.5 (± 1.33)	0.189		
	downstream T _{end}	15.7 (± 0.98)			

Where: T₀- freshly collected river water/sediment extracts; T_{end}- river water and sediment extracts after PNP biodegradation. Number of TRFs obtained with labelled primer 1087R – VIC; ± standard error of the mean (S.E.M.), n=3.

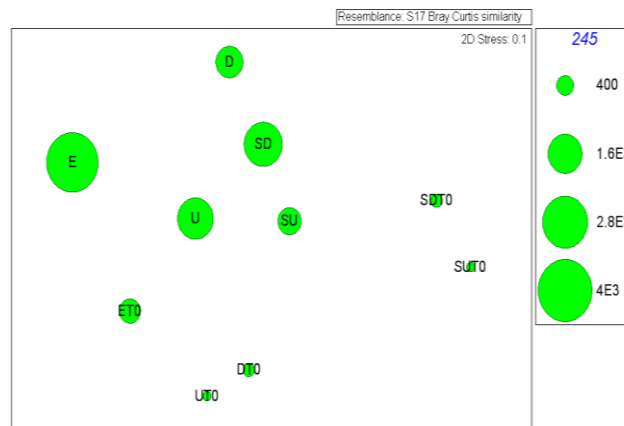
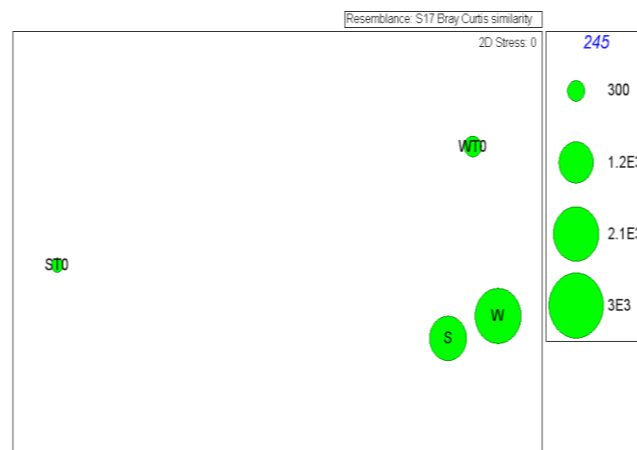
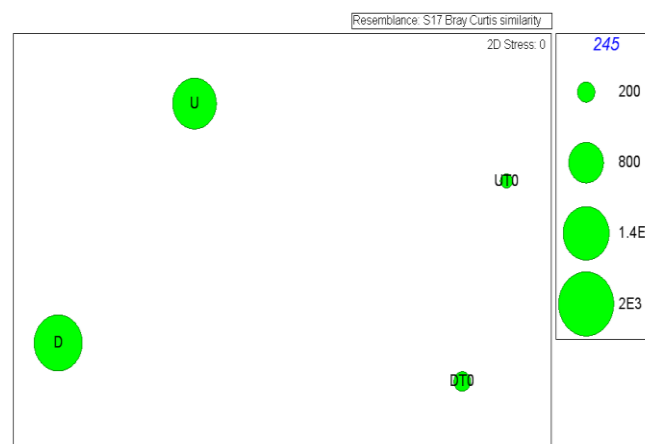
a**b****c**

Figure 3.5: A 2D bubble plot presenting the amount of TRF 245 nt (TRF identified as *Pseudomonas syringae*) using Bray-Curtis similarity. The area of green circles corresponds to the amount of TRF 245 nt present in environmental samples. Figure legend: **a**- averaged treatments (time vs. location vs. material), stress 0; **b**- material (water vs. sediment), stress 0; **c**- location (upstream vs. downstream), stress 0.1; where: **U**- upstream, **E**- effluent, **D**- downstream, **S**- sediment, **W**- water, **T₀**- before incubation, **without T₀** – after complete PNP biodegradation.

Table 3.6: ANOVA for relative abundance of TRF 245 nt in bacterial TRFLP profiles.

Variable	TRFLP profile	Abundance % TRF 245 nt	ANOVA P-value	
Treatment	upstream water T ₀	1.54 (± 0.77)	0.018	0.007
	effluent water T ₀	10.76 (± 2.84)		
	downstream water T ₀	4.27 (± 0.32)		
	upstream sediment T ₀	3.51 (± 1.79)		
	downstream sediment T ₀	5.71 (± 0.38)		
	upstream water T _{end}	21.90 (± 10.16)	0.446	
	effluent water T _{end}	33.10 (± 3.01)		
	downstream water T _{end}	21.30 (± 11.55)		
	upstream sediment T _{end}	12.10 (± 3.00)		
	downstream sediment T _{end}	32.10 (± 10.39)		
Material	water T ₀	5.52 (± 2.14)	0.676	0.069
	sediment T ₀	4.61 (± 0.57)		
	water T _{end}	25.69 (± 5.44)	0.661	
	sediment T _{end}	22.08 (9.27)		
Location	upstream T ₀	2.52 (±0.98)	0.041	0.126
	downstream T ₀	4.99 (± 0.57)		
	upstream T _{end}	17.00 (± 3.94)	0.288	
	downstream T _{end}	27.10 (± 8.41)		

Where: T₀- freshly collected river water/sediment extracts; T_{end}- river water and sediment extracts after PNP biodegradation; ± standard error of the mean (S.E.M.); n=3.

3.4.5. Quantification of *pnpA* and *mar* functional marker copy number by QPCR

The QPCR analysis was performed to determine the copy numbers of functional marker *pnpA* (**Figure 3.6 a**) and *mar* (**Figure 3.6 b**) in different environmental samples before and after PNP biodegradation. The ANOVA was performed for the copy number of *pnpA* to compare different time points, as well as treatments. Overall across all treatments there was an increase in the copy number of *pnpA* after PNP biodegradation (except for effluent), and the differences were found in copy number of *pnpA* between treatments, since downstream sediment had significantly higher *pnpA* copy number after PNP biodegradation in comparison with other treatments. QPCR for *mar* gene indicated that the copy number determined for *mar* had increased in all environmental samples after PNP biodegradation (T_{end}). Higher copy number of *mar* was observed in effluent at T_0 and T_{end} and in sediment downstream at T_{end} . In addition, the copy numbers determined for the 16S rRNA gene were significantly higher in sediment downstream at T_0 (**Figure 3.6 c**). Lower 16S rRNA gene copy numbers were observed after PNP biodegradation (T_{end}) across all treatments (except for effluent).

However, the non-target hits were present in the copy number for *pnpA*, counts for *mar* does not represent PNP-degraders only, and the counts are very low for 16S rRNA gene (possibly due to the long gene fragment). This indicates that QPCR results do not represent the actual abundance of bacteria in the samples.

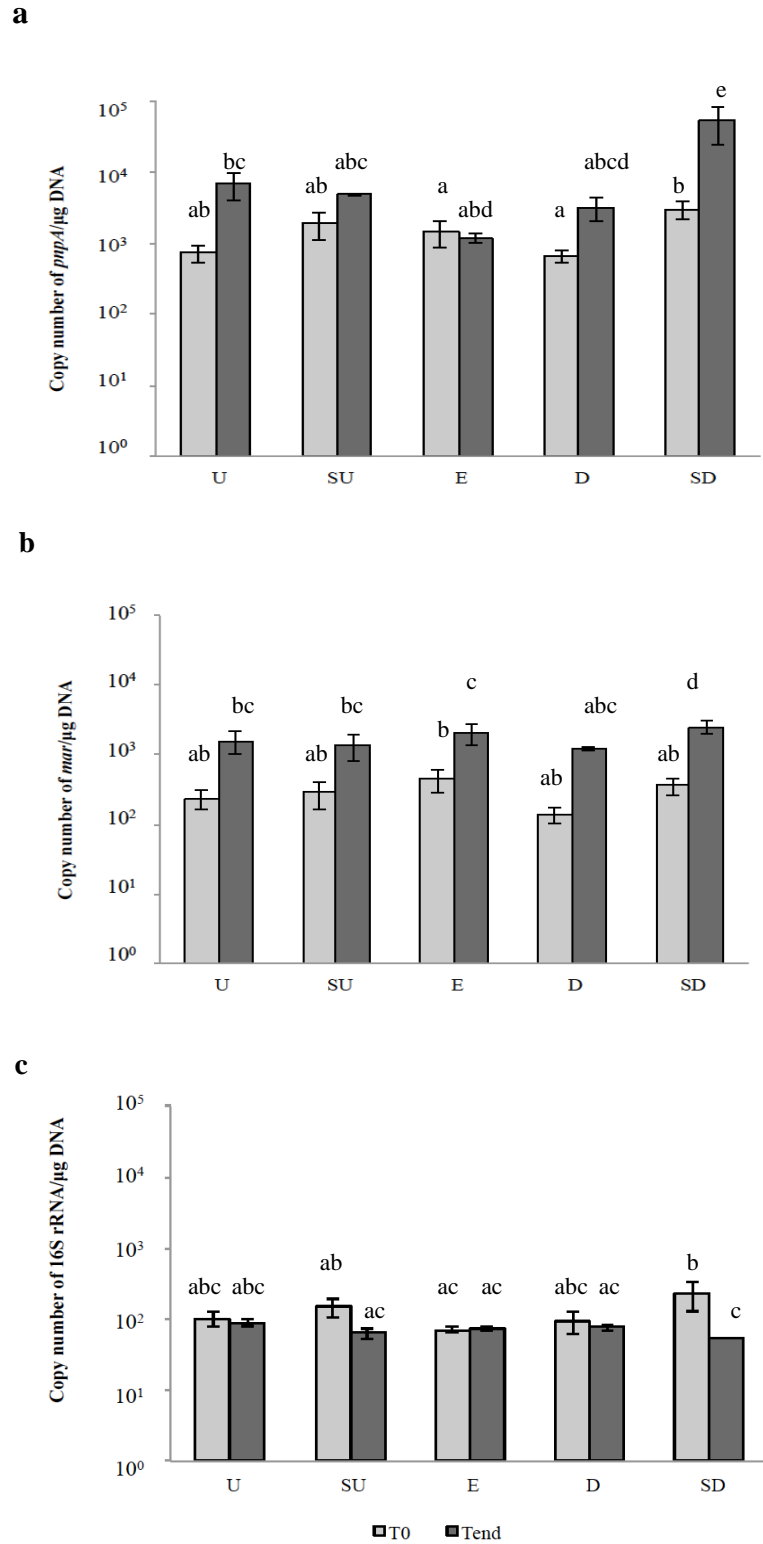


Figure 3.6: QPCR for functional genes and 16S rRNA gene.

Where: **a**-*pnpA*; **b**-*mar*; and **c**- 16S rRNA gene in environmental samples before (T_0) and after PNP biodegradation (T_{end}); **U**-river water upstream, **SU**-sediment extract upstream, **E**-effluent from Wellesbourne WWTP, **D**-river water downstream, **SD**-sediment extract downstream; Treatments with different letters a, b, c, d, e are significantly different ($P < 0.05$) while treatments with more than one letter e.g. ab, ac, abc, abcd, bc are similar to each other; error bars show \pm standard error of the mean (S.E.M), $n=3$.

3.5. Discussion

3.5.1. Effect of inoculum material and location on the biodegradation of PNP and microbial community structure in river water and sediment

Based on ANOVA for PNP biodegradation rate (**Figure 3.2, Table 3.1**), and number of PNP degraders (**Figure 3.3, Table 3.4**) it can be suggested that inoculum material and its origin did not significantly affect the biodegradation potential, and therefore, had no effect on the outcome of PNP biodegradation experiments. Although, the initial chemistry of inoculum (river water and sediment extracts) was significantly different in terms of pH, and concentration of ammonia, nitrite and nitrate and bacterial community structure, it did not affect the PNP biodegradation potential. These findings would support the initial hypothesis, which assumed that the type of inoculum material and the location at which it is sampled does not affect inoculum potential to biodegrade chemicals. However, ANOVA was not suitable statistical analysis for PNP biodegradation data due to high variability of replicates. It was found that there were few statistical differences between samples because their replicates varied greatly not because samples were similar. Therefore, it was difficult to determine whether there are any differences between samples or not. Analysis of such variable data would require data transformation and non-parametric tests.

Some evidence exists that inoculum type does not matter for chemical biodegradation. For instance, Bradley *et al.*, (2001) investigated the biodegradation of methyl-*tert*-butyl ether (MTBE) by stream and lake bed sediments collected from 11 sites throughout the United States. It was found that MTBE-degrading microorganisms are widespread in the environment and that mineralization of MTBE did not differ significantly between sediments collected from MTBE contaminated sites and from sites with no history of MTBE exposure. Also, Jürgens *et al.* (2002) reported no variation of estrogen biodegradation potential along the same river and between different rivers for water and sediment samples. However, a few authors have pointed out the importance of the selection of an appropriate inoculum material to obtain reliable biodegradation results. This was proved by Vargas *et al.* (2006) in the biodegradation of nitrophenol in an anaerobic-aerobic sequencing batch reactor (SBR) with two types of inocula: aerobic and anaerobic sludge. Although, there was

no difference in acclimation times and stability of the process between the two inocula, the originally anaerobic inoculum was more effective in the nitrophenol biodegradation. Application of inocula sampled from one environmental compartment does not necessarily lower the risk of test failures. Mezzanotte *et al.* (2005) indicated low reproducibility of biodegradation tests carried out with activated sludge sampled at different times from the same WWTP. Furthermore, the influence of different types of inocula as well as the amount of inoculum (microbial biomass) on the biodegradation pattern of acetate, 4-nitrophenol, and the three methoxyaniline isomers was investigated by Kool (1984). While river sediment could not degrade 4-nitrophenol, an inoculum from garden soil gave 60% degradation and effluent of an activated sludge plant was able to degrade 4-nitrophenol completely at a concentration of 19 mg/L (Kool, 1984).

Variation between replicates has been also observed for TRFLP bacterial profiles. The fact that one replicate was different to the others demonstrates the difficulty of sampling relatively rare organisms in relatively low concentration of biomass. Interestingly, the ANOSIM and MVDISP analysis for TRFLP bacterial community profiles obtained in the present study indicated that the bacterial community composition was affected by the inoculum material (river water and sediment TRFLP profiles were different (**Figure 3.4**)) and location from which the inoculum was sampled. These results were opposed to the initial hypothesis that the sampling site location does not affect the inoculum community structure, and that inoculum material does not impact on bacterial community composition. Some authors reported consistent composition of microbial communities among inocula with different origin. For instance, Xia *et al.* (2010) evaluated microbial community composition in five municipal biological WWTP reactors in China and the United States, and found that all communities contained a core set of species with almost identical compositions, and were present in the same proportion in all samples. In contrast, Moreno-Andrade and Buitrón (2004) observed the significant variations in the specific methanogenic activity of tested inocula based on their microbial composition. Tested inocula had different origins including an anaerobic reactor degrading wastewater of a brewery (BRW), an anaerobic reactor of a chemical industry degrading terephthalic acid (CHE), a brewery industry upflow anaerobic sludge blanket (UASB), fresh cow manure (COM), and an activated sludge treatment

plant (ASL); and the highest microbial activity was observed for the inoculum from UASB (Moreno-Andrade and Buitrón, 2004).

3.5.2. Effect of effluent discharge on the biodegradation of PNP and microbial community structure in river water and sediment

Bacterial communities present in WWTP have contact with a variety of chemicals and are involved in their biodegradation (Martins *et al.*, 2011). Consequently, effluent discharged from WWTP may introduce some active bacterial species from WWTP into the streams, and therefore, increase their abundance downstream of the effluent outfall (Goñi-Urriza *et al.*, 1999). Based on that, it was initially hypothesised, that effluent discharged from Wellesbourne WWTP would affect (improve) the biodegradability potential of inocula collected downstream of the effluent outfall, since bacterial populations which are more adapted to organic chemicals were expected to be more abundant in the downstream river water and sediment samples. Experimental results in the current work indicated that effluent from Wellesbourne WWTP had no impact on the presence of specific degraders (**Figure 3.3**) and river water microbial community structure (**Figure 3.4**), and it did not affect the number of copies of either functional markers for PNP biodegradation (**Figure 3.6**) or the biodegradability potential of downstream inocula. Wellesbourne WWTP is the largest WWTP discharging treated effluent directly to River Dene and the population connected (receiving population) to this WWTP was 6,924 people in 2010. According to the Severn Trent Water monitoring data the average effluent flow from Wellesbourne WWTP, on the sampling day, was 17.52 L/sec. The quality of that effluent was acceptable with respect to the levels of ammonia, nitrate, nitrite and DOC which should be within the range of 19.7, 10, 1.0 and 20 mg/L, respectively (DWI, 2012; US EPA, 2012; NERRS, 2012). Good effluent quality could explain why there was no impact of Wellesbourne WWTP outfall on the microbial community composition in the River Dene downstream of effluent outfall and why it did not affect the rate of PNP biodegradation with inoculum collected downstream of WWTP. In case of bad effluent quality and high concentration of toxic chemicals, the effluent discharge would lead to increased BOD in river water and disturbance of naturally occurring bacterial populations downstream of the

effluent outfall. This could result in the weakened or lost potential of these populations for biodegradation of chemicals.

No presence of *npdA2* gene was observed in river water, sediment or effluent samples which might suggest that this gene does not play a key role in the biodegradation of PNP with the described inocula. It is also possible that the developed functional marker approach did not enable efficient detection of gene *npdA2* in environmental samples used in this study. QPCR analysis revealed that a higher number of copies of the *mar* functional marker were present in the effluent at T₀ in comparison with other types of inoculum (**Figure 3.6 b**), but they were not significant. Additionally, QPCR results were found to be unreliable for determination of abundance of PNP-degraders due to false positive results (as discussed in Chapter 2). TRFLP showed that *P. syringae* (TRF 245 nt) was present in river water and sediment treatments and a significantly higher abundance of TRF 245 nt was in effluent samples (**Figure 3.5** and **Table 3.6**). This could be due to the nature of effluent, which is discharged from Wellesbourne WWTP after the treatment. Microorganisms which already had contact with a variety of organic chemicals present in wastewaters collected and treated at Wellesbourne WWTP could be present in the effluent discharged into the River Dene and which was expected to have an effect on downstream community structure as discussed above. Contact of bacterial communities with a chemical may affect the biodegradation potential of tested inocula. Viñas *et al.* (2002) tested the potential of three enriched inocula to degrade different fractions of crude oil, and how the enrichment procedures with selected oil products can provide metabolically specialized microbial consortia. Results showed that obtained microbial consortia efficiently degraded the class of hydrocarbons used as a carbon source in their respective enrichment procedures. Additionally, the extent of the attack on the different fractions of a crude oil by the inocula was consistent with their origin (Viñas *et al.*, 2002).

In order to test the hypothesis that the effluent discharge does not affect composition of microbial communities the ANOSIM was performed for TRFLP bacterial profiles from environmental samples. Bacterial community structure appeared to be similar in river water upstream and downstream of the effluent outfall and river water from effluent discharge point did not have a different community composition than the

downstream site. This may suggest that WWTP effluent did not introduce any new bacterial strains capable of PNP biodegradation and the effluent discharge did not disturb the existing microbial populations in downstream river water and river sediment. Therefore, the initial hypothesis that effluent discharge does not affect downstream bacterial communities was supported. Although, the presence of other WWTPs further upstream of Wellesbourne WWTP could suggest that effluent discharge from those WWTPs could impact on the bacterial community present in river water collected upstream of Wellesbourne WWTP. Hence, no differences were found in microbial populations present at the effluent and downstream site.

It could be speculated that lack of differences in the potential for PNP biodegradation of river water and sediment bacterial communities from different sites could be due to their possible pre-exposure to e.g. pesticides and their metabolites which might have run-off from the nearby fields. Another possibility is that the location of sampling points were not distant enough to each other, since they are located within a small stretch of River Dene, which is downstream of effluents discharged to River Dene from other than Wellesbourne WWTPs. It is noteworthy that there are three WWTPs located directly upstream of Wellesbourne WWTP (Chapter 1, **Figure 1.9**). Ettington and Combrook WWTPs have receiving population of 894 and 78 people, respectively, and the effluent is discharged to one of the tributaries of River Dene. The third WWTP is located in Butlers Marston with the receiving population of 556 people and effluent is discharged directly to River Dene. Since the river water already had contact with effluent upstream it could be suggested that similar chemical-degrading populations were abundant upstream of Wellesbourne WWTP effluent discharge and downstream of that point and this could explain why there was no difference between the sampling sites used in this study.

The present experimental design also did not involve direct sampling of effluent water which could be the source of different microbial populations than those present in river water mixed with effluent. It might be speculated that the effluent was diluted in river water to such an extent that the detection of less abundant species was not possible. Future experimental work could involve sampling of river water and sediment at different distances downstream of a WWTP effluent discharge point and further upstream of that point including sites located on different river stretches. It is noteworthy that the downstream sampling site in the current experiment was

located 530 m from the effluent discharge point which could have been well below the river water and effluent mixing zone. Additionally, biodegradation tests could be conducted with bacterial inocula collected from truly pristine and anthropogenic sites, to investigate the occurrence of PNP-degrading bacterial communities in the environment.

Variability of inocula can be the source of inconsistent results in biodegradation tests. Inoculum source is important, because it can affect the amount of bacterial biomass (Kool, 1984) and the presence of specific degraders which are introduced into the test systems (Szabó *et al.*, 2007; Thouand *et al.*, 2011). However, in this project similar PNP-degrading bacteria were isolated from river water, sediment and effluent samples. Also, the present study revealed no correlation between inoculum size and PNP biodegradation rate, although it was difficult to determine whether inoculum material and sampling site affected the potential of the inoculum to degrade PNP. Nevertheless, the bacterial community structure in tested inocula was affected by inoculum material as the bacterial community profiles of water and sediment extracts had different communities. The accurate prediction of chemical biodegradation is still challenging, and because it depends on the inoculum, it is recommended as good practice to characterize inocula during biodegradation experiments. Determination of inoculum size and microbial community composition was found useful in the current project since it enabled better comparison of test results. Therefore, these procedures might be considered as a standardized method for setting up biodegradation tests with variable inocula, an aspect that is missing in OECD studies used to characterise chemical persistence by Industry and Regulatory bodies.

3.6. Conclusions

Based on the results obtained in this study the following conclusions can be drawn:

1. High variability of replicates within treatments caused difficulties with statistical analysis, and therefore, it was hard to determine how the biodegradation of PNP was affected by the type of inoculum material and the location from which it was collected on the River Dene. However, the microbial community composition was significantly affected by the inoculum material (river water, sediment extract) and by the location of sampling point.
2. The abundance of *Pseudomonas syringae* 245 nt increased during PNP biodegradation in congruence with results described in Chapter 2, suggesting that *P. syringae* was a major driver of PNP biodegradation.
3. Higher abundance of *P. syringae*, as determined by abundance of TRF 245 nt in inoculum, does not necessarily reflect the inoculum's potential for PNP biodegradation.
4. There was a significant impact of inoculum type, in terms of sampling location and material on the number of copies of *pnpA* and *mar* after PNP biodegradation.
5. Effluent does not impact on river water bacterial community structure, since no significant effect of effluent outfall from the WWTP was found on bacterial communities downstream of the outfall.

Chapter 4

Effect of inoculum density and diversity on *para*-nitrophenol biodegradation

4.1. Introduction

Established by the Organisation for Economic Cooperation and Development (OECD), biodegradation test guidelines are used in the environmental risk assessment for chemicals to predict chemical persistence and possible ecotoxicological impacts on the natural ecosystem. The biodegradation tests usually involve a variety of environmental samples that are used as inocula and are collected from natural and man-made compartments throughout the year (Doi *et al.*, 1996; Reuschenbach *et al.*, 2003; Yuan *et al.*, 2004).

Biofilms are used as inocula in the biodegradation simulation test guideline (OECD 303 b) and bioreactors with biofilms have been applied to study the bioremediation of a variety of chemicals e.g. chlorophenols (Puhakka *et al.*, 1995; Kargi and Eker, 2005), herbicides (Oh and Tuovinen, 1994), azo dyes (Zhang *et al.*, 1995), and heavy metals (Diels *et al.*, 2003, Chang *et al.*, 2006). It is known that high species richness (Sabater *et al.*, 2007), biofilm resistance to toxic chemicals e.g. antimicrobial agents (Mah and O'Toole, 2001) and recovery potential (Morin *et al.*, 2007; Proia *et al.*, 2010) make river biofilms a good inoculum source for chemical biodegradation tests. Moreover, biofilms form on most surfaces exposed to water and thus are fundamental components of aquatic ecosystems. Biofilm microorganisms are the basis of aquatic food webs, and it is known that bacteria residing within biofilms play a key role in the biodegradation of organic chemicals and in biogeochemical cycles (Brümmer *et al.*, 2003; Chénier *et al.*, 2003). The microbial biofilm matrix induces a number of changes on the physical and biological properties of sediment environments, which may range from the stabilization of sediments to the stabilization of microbial populations within biofilms (Decho, 2000). Moreover, river biofilms also exchange so-called suspended particulate matter (SPM) with the

water phase and represent a combination of planktonic and sessile communities (Brümmer *et al.*, 2003). Stream biofilms represent dynamic matrices of microbial communities that undergo changes in the composition of photosynthetic periphyton, heterotrophic microbes, and extra-cellular polymers (EPS) in response to environmental conditions (Palmisano *et al.*, 1991; Writer *et al.*, 2011). For instance, Lawrence *et al.*, (2009) reported that the biofilm communities developing under the influence of TCS and triclocarban (TCC) had community architecture and composition different from either control or nutrient amended biofilms.

Microbial inocula are subjected to seasonal variability due to the changes of environmental conditions which shape the natural ecosystems and affect processes occurring in environmental compartments (Bertram, *et al.*, 2001; Yunus and Nakagoshi, 2004). For example, changes in river water volume, and flow rates due to storm events lead to disturbance of biota (e.g. plants, fish) and microbial communities within biofilms present in rivers (Hudon, 1997; Hunt and Parry, 1998; Midwood and Chow-Fraser, 2012). Changes in the levels of river water also affect the concentrations of available nutrients as well as chemicals. Lower water levels lead to much higher concentration of chemicals (Loraine and Pettigrove, 2005). Conversely, a high level of water in the river causes the dilution of chemicals and nutrients but increases the amount of suspended matter in river water and more dynamic mixing, which may affect microbial populations (Naudin *et al.*, 2001). The biofilms decay with time mainly due to seasonal conditions and higher stream velocities and changes in the surface-to-volume ratio (Brümmer *et al.*, 2003; Writer *et al.*, 2011) which may affect the total metabolic activity of biofilms and carbon cycling in rivers (Brümmer *et al.*, 2003). Changes in temperature also affect bacterial biomass production (LaPara *et al.*, 2000), and varying light conditions throughout the annual cycle impact on algal and bacterial community composition, especially the ratio between algae and bacteria within biofilms (Romaní and Sabater, 1999). As a consequence of variable environmental conditions, microbial communities present in rivers are impacted and their composition and function may be altered (Brümmer *et al.*, 2003), which can affect the overall potential for chemical biodegradation i.e. self purification processes. For example, seasonal variations in stability of 2,4-dichlorophenoxyacetic acid (2,4-D) were reported by Watson (1977) in river water and mud samples, and the maximum breakdown of 2,4-D was observed during the

winter flood conditions. This could be due to the fact that nutrients, chemicals and bacteria are adsorbed on the particles of suspended sediment, and therefore, enhance the biodegradation processes in rivers (Nesbit and Watson, 1980).

Since bacterial density and biomass in the aqueous environment varies seasonally (Velimirov and Walenta-Simon, 1992) it affects the inoculum concentration at a given location (Vázquez-Rodríguez *et al.*, 2006). Also, the physicochemical characteristics of river water may affect bacterial growth and function of microbial communities present in biofilm and river water inocula (Reardon and Zhang, 1992; Sabater *et al.*, 2002). It has been reported that microbial community structure matters for community function (Carlson *et al.*, 2004; Johnson *et al.*, 2004; Kirchman *et al.*, 2004) and changes in microbial populations can lead to disruption or even loss of function in natural ecosystems.

The OECD biodegradation guidelines are vague about the methods for inoculum collection. Although it is known that the inoculum density (microbial biomass) is an important factor affecting the biodegradation of chemicals (Thouand *et al.*, 1995) it is not well documented what drives the spatial and seasonal variation of microbial inocula. Variable inocula represent an important issue in biodegradation studies (Courtes *et al.*, 1995; Mezzanotte *et al.*, 2005), and inconsistent test results may exist due to biodegradation failures with inocula sampled at different times of the year. Consequently, reproducibility of biodegradation tests may also be impacted by the microbial inoculum and seasonal changes. It is not known how the application of river biofilm inocula could improve the biomass level in the biodegradation test systems and improve the reproducibility of biodegradation results.

4.2. Objectives

The aim of this study was to investigate the effect of sampling date, location and inoculum material on the reproducibility of *para*-nitrophenol (PNP) biodegradation tests which were carried out in November 2011, February and May 2012. The following hypotheses were tested: sampling date, location and inoculum material affect (1) inoculum quality and quantity in terms of bacterial community composition and biomass within river water and river biofilms; and (2) inoculum potential for PNP biodegradation which is related to the diversity of PNP functional markers *pnpA* and *mar*.

4.3. Materials and methods

4.3.1. River Dene biofilms

Six biofilm traps were prepared, and each consisted of Hole Airbrick (21.0 x 13.5 x 5.0 cm) (Travis Perkins Trading Co. Ltd, UK) with 11 Polysine glass slides (5.7 x 2.5 cm) (VWR, International) per brick (**Figure 4.1 a**), which were attached with cable ties (165 x 2.6 mm) (BHGS Ltd, UK). At each sampling site located upstream, at effluent outfall and downstream of that point, described in Chapter 3, two biofilm traps were placed on the bed of River Dene. Biofilm traps were left in the river for eight days to allow colonisation of the slides (total surface area of 28.5 cm²) by microorganisms and development of river water biofilms (**Figure 4.1 b**). The first samples were taken in November 2011 and further samples were collected in February and May 2012.

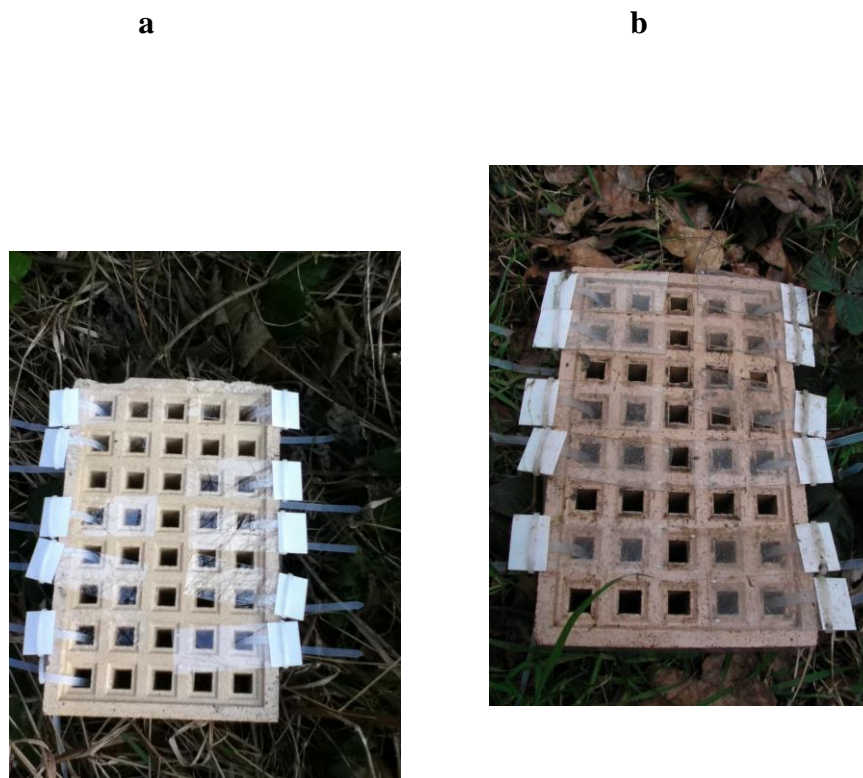


Figure 4.1: Biofilm trap consisting of brick with attached glass slides.

Figure legend: **a**- biofilm trap before biofilm colonisation, **b**- biofilm trap after 8 days of colonisation in River Dene.

4.3.2. The biodegradation of PNP by river biofilms

In order to test biofilms potential to PNP biodegradation treatments were prepared in triplicates per sampling site and consisted of sterile Duran Schott bottles (500 mL) (Fisher Scientific, UK) with 300 mL of sterile river water inoculated with three slides colonised by river water biofilms. Sterile river water was prepared by autoclaving at 121°C, for 15 minutes, at 1.1 atm water samples collected from River Dene at three sampling points. Positive control consisted of sterile river water inoculated with induced PNP-degrading isolate *Pseudomonas syringae* AKHD2, grown on LB medium with PNP (56 mg/L) for 48 hours at 25°C. Sterile river water was used as a negative control. PNP (dissolved in water) was added as a sole source of carbon to the initial concentration of 2 mg/L. The incubation conditions were as described in Chapter 2. Every two days, 1 mL aliquots of water were taken and used for HPLC measurement using method described in Chapter 2 section 2.3.3.

4.3.3. Biofilm biomass

In order to determine the amount of biofilm biomass developed on the glass slides, the amount of collected biofilm was measured using three different assays: extraction of total carbohydrates, bacterial counts and DNA extraction.

4.3.3.1. Total carbohydrates extraction

The total biofilm carbohydrate concentration (extracellular polysaccharides (EPS)) was measured in biofilms developed on the glass slides. Triplicate biofilms were scraped per sampling site, from both sides of the slides using a sterile nylon brush, followed by a wash of the slide with 5 mL of sterile distilled water. Aliquots of 2 mL of biofilm biomass suspension were used for the phenol-sulphuric acid assay (Dubois *et al.*, 1956), where 1 mL of 5% aqueous phenol solution (wt/vol) (Phenol Liquid, Fisher Scientific, UK) was added followed by 5 mL of concentrated

sulphuric acid. The tubes were allowed to stand for 10 minutes at room temperature, and then were shaken to mix the content of the tubes and incubated for 20 minutes at 30 °C before the readings were taken. Absorbance of developed colour was measured at 458 nm, using Unicam 5625 UV/VIS Spectrophotometer (ATI Unicam, UK). The amount of extracted carbohydrates was expressed as glucose equivalents in µg/mL, based on the calibration standard curve obtained with prepared solutions of D-glucose (Fisher Scientific, UK) in sterile distilled water. The range of glucose concentration was from 5 to 500 µg/mL.

4.3.3.2. Bacterial viable count

The number of cultivable bacteria present in collected biofilms was determined and used as an indication of potential inoculum size. Aliquots of suspended biofilm biomass (obtained as described for carbohydrates extraction) were diluted in series of 10-fold dilutions and spread plated onto R2A agar plates, followed by incubation for 5 days at 25 °C. After this time, plates were examined and the number of bacterial colonies was determined and expressed as number of colony forming units per mL (cfu/mL).

4.3.3.3. DNA extraction from biofilms

Before incubation (T_0) and after complete PNP biodegradation or at the end of the incubation (T_{end}), biofilms were harvested in triplicates from three slides per sampling site via three rounds of scraping according to Deines *et al.* (2010), using a sterile nylon brush, followed by washing of the slides each time with 15 mL aliquots of sterile distilled water. The 45 mL of suspended biofilm biomass collected was filtered using a vacuum filtration system with Millipore GVWP04700, (hydrophilic Durapore) polyvinylidene fluoride membrane filters (0.22 µm mesh, 47 mm diameter), attached to a vacuum source. Membrane filters with concentrated biofilm biomass were folded and placed in the bead tubes of the Power Water DNA extraction kit (MoBio, UK). DNA extraction from biofilms was performed according

to manufacturer's protocol. The DNA concentration was measured using a NanoDrop ND-1000 Spectrophotometer (Thermo Scientific Fisher, UK).

4.3.4. Microbial ecology methods

The PCR reactions, TRFLP, PNP functional genes amplification and QPCR were performed as described in Chapter 2 sections **2.3.9** and **2.3.15**, respectively. Additional analysis for TRFLP profiles was performed using option BEST in Primer 6. Environmental variables (temperature, river water height, nitrite concentration and river discharge area), which best match the multivariate pattern of TRFLP profiles, were selected for BEST analysis to find the correlation between these variables and bacterial TRFLP profiles. Correlations were determined based on the comparison of two matrixes, one for TRFLP profiles and one for environmental variables.

4.3.5. Seasonal conditions

After biofilm traps were removed from the River Dene, light, temperature as well as river flow measurements were performed *in situ*. Samples for water quality were also taken.

4.3.5.1. Light and temperature

Light intensity was measured at the surface of river water at each sampling site using a SKP 200 Quantum light meter (Kye Instruments, USA) range 0-20000 μmol s. Temperature river water was measured at each sampling site using a Mini Thermometer 0560 1110 (Testo, UK).

4.3.5.2. Water quality

Two aliquots of 50 mL of river water were collected along with river water biofilms from each sampling point and analysed for the levels of DOC, ammonia, nitrite, nitrate, COD and pH. The measurement of ammonia, nitrite, nitrate and COD were performed using HACH, LANGE cuvette tests (LCK303 and 304, LCK342, LCK339 and 340, LCK614 and 914) according to manufacturer's protocol. All measurements were performed using DR3800 Bench Spectrophotometer (HACH LANGE, USA). DOC was determined using kit and Shimadzu TOC-V Total Organic Carbon Analyser. Analysis of total carbon (TC) was based upon the combustion method. The TC combustion tube was filled with platinum catalyst and heated to 680°C. Carrier gas (purified air or oxygen) was supplied to this tube. A sample was injected and all the carbon oxidised to CO₂ which was measured using an infra red analyser. Analysis of total inorganic carbon (TIC) was based upon chemical oxidation. A sample was introduced to an IC reaction chamber containing 25% phosphoric acid. Total organic carbon (TOC) was measured by using a 'by difference' method whereby TC of a sample is measured then the TIC, and the difference being the TOC. Measurements for dissolved organic carbon (DOC) were obtained by filtration through 0.45 µm Millipore filters. All measurements were performed at Safety and Environmental Assurance Centre (SEAC) Unilever (Bedfordshire, UK).

4.3.5.3. Flow rate

The river flow rate, water depth, discharge area and total discharge of the River Dene were determined (single measurement) at each sampling site, using the open channel flow meter, Model 801 (Valeport, UK) from the School of Engineering, The University of Warwick, UK.

4.3.6. Statistical analyses

4.3.6.1. Analysis of biodegradation and QPCR data

The time taken for PNP biodegradation (DT-values) and ANOVA for DT-values and ANOVA for QPCR data were calculated from triplicates of treatments using GenStat 13th edition, as described in Chapter 2 section 2.3.18.1. Correlations were calculated using function CORREL in Microsoft Excel. Correlation coefficients were compared with Critical Values of the Pearson Product-Moment Correlation Coefficient table to determine whether there is a statistically significant relationship between compared factors at $P < 0.05$.

4.3.6.2. Analysis of TRFLP profiles

ANOVA was performed using GenStat 13th edition, and Primer 6 was used for analysis of bacterial TRFLP profiles (NMDS and ANOSIM) as described in Chapter 2 section 2.3.18.2.

4.3.6.3. Analyses for core and satellite species from TRFLP profiles

4.3.6.3.1. Analysis of the local community

Each biofilm bacterial TRFLP profile was treated as a local community. Rank-abundance plots were used to determine differences in bacterial local community structure for each sampling site (upstream, effluent, downstream), sampling date (November, February, May) and incubation time point (T_0 and T_{end}). For each sample the relative abundance of each taxon (TRF peak area) was standardized to per cent values before construction of the rank-abundance plots. The rank-abundance plots were visualized by plotting the taxa rank order on the x-axis against relative abundance (\log_{10} transformed) on the y-axis. For each plot a linear regression model was fitted, represented by the equation, $\log_{10}y = a + bx$, where a is the intercept and b is the slope of the plot. The slope (b) was used as a descriptive statistic for changes in community structure (Ager *et al.*, 2010). Linear regressions, coefficients of determination (r^2) were calculated using Microsoft Excel, and significance (P) and one-way ANOVA were calculated using GenStat 13th edition.

4.3.6.3.2. Analysis of the metacommunity

All TRFLP profiles from biofilms obtained in November, February and May from each time point (T_0 or T_{end}) were treated as metacommunities. The two-group core-satellite modeling approach was applied to the bacterial metacommunities to (1) establish whether the PNP-degrading *Pseudomonas syringae* (previously identified as TRF 245 nt) belong to core or satellite species, (2) determine whether distribution and persistence of *P. syringae* was affected by sampling date, and (3) investigate how this correlated with PNP biodegradation. The TRFs in each metacommunity were divided into core and satellite groups by decomposing the overall distribution using the ratio of variance to the mean abundance for each bacterial taxon (van der Gast *et al.*, 2011). The variance to mean ratio, or index of dispersion, is an index used to model whether species follow a Poisson distribution, falling between 2.5% and 97.5% confidence limits of the χ^2 distribution. Bacterial TRFs that occurred only once in TRFLP profiles were excluded from this analysis, as their dispersion in space would have no variance. Poisson distribution tests were carried out according to the method described by Krebs (1999). Rank-abundance plots with core and satellite species were visualized by plotting the taxa rank order on the x-axis against mean % relative abundance on the y-axis with \log_{10} scale (Rogers *et al.*, 2013). An Excel macroprogram was applied to compare the slopes of rank-abundance plots with core and satellite species between metacommunities to determine the significance of differences in metacommunity structure between sampling dates and incubation time points. Regression analysis, coefficients of determination (r^2), residuals and significance (P) were calculated using Minitab software (version 15, Minitab, University Park, PA, USA).

4.4. Results

4.4.1. The biodegradation of PNP

No PNP biodegradation (**Figure 4.2**) was observed for upstream and downstream river water biofilms collected in November 2011, while two out of three replicates of effluent biofilm completed PNP biodegradation within nine days (**Figure 4.2**). Complete PNP biodegradation was recorded after 11 and 13 days for biofilms collected in February and May 2012, respectively. ANOVA revealed that there was a significant difference for DT values between effluent biofilms collected in November 2011 and February 2012 (**Table 4.1**). An effect of inoculum material on PNP biodegradation was also observed. Effluent biofilms showed consistent PNP biodegradation rates which had a significantly longer incubation time than effluent river water. Although, upstream and downstream biofilms had similar DT values for PNP biodegradation, significantly slower PNP biodegradation was recorded for effluent biofilms in February and May 2012.

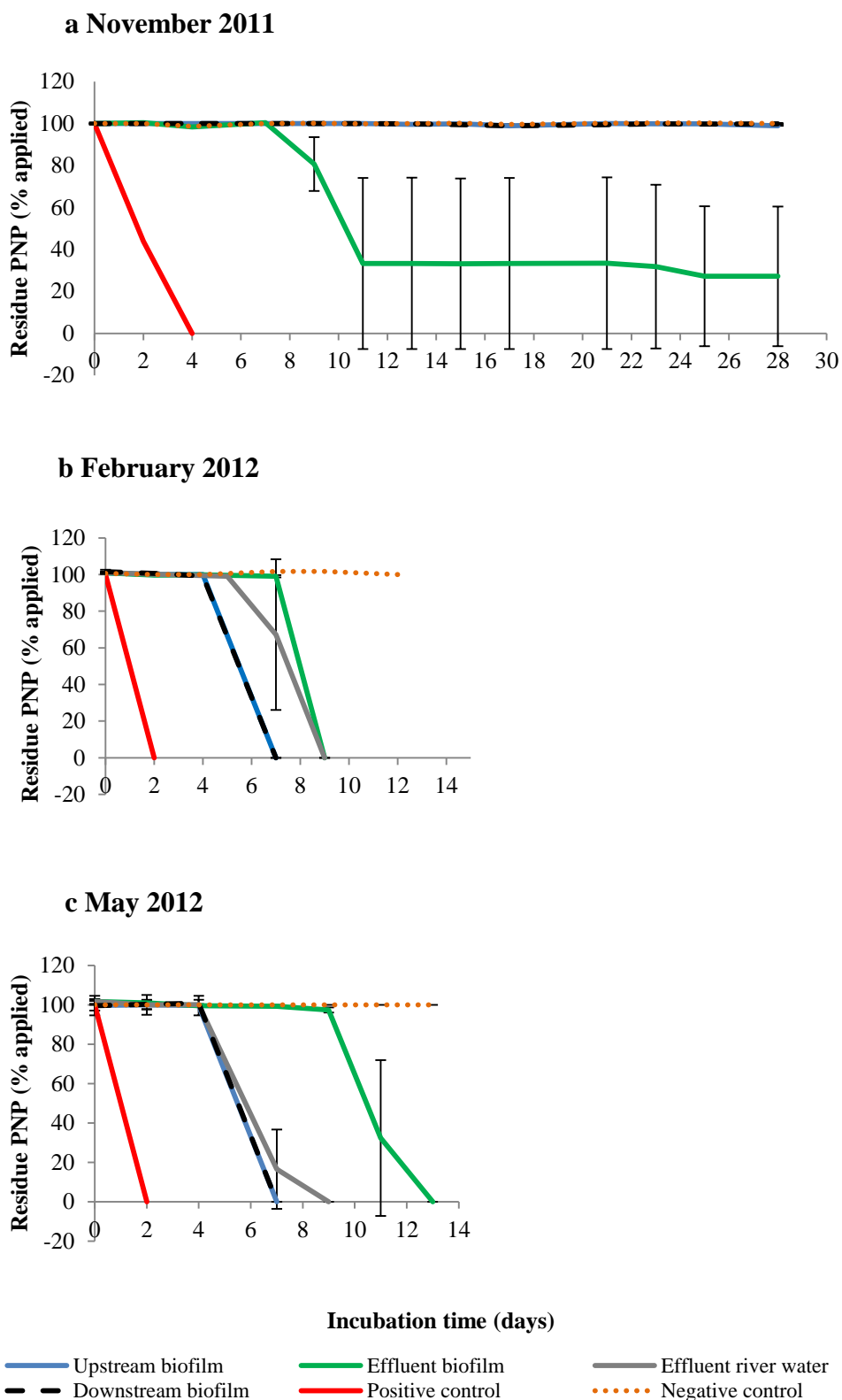


Figure 4.2: The biodegradation of PNP with biofilms and river water.

Figure legend: **a**- inoculum collected in November 2011, **b**- February 2012, and **c**- May 2012. Average values from treatments in triplicates. Error bars showing standard error of the mean (S.E.M.), n=3.

Table 4.1: DT-values calculated for PNP biodegradation with biofilms and river water.

Date	Location	DT ₁₅	DT ₅₀	DT ₉₀
November 2011	Upstream	ND	ND	ND
	Effluent	14.00(±5.24) ^{ac}	16.11(±5.95) ^a	17.62(±5.19) ^a
	Downstream	ND	ND	ND
February 2012	Upstream	4.50(±0.00) ^b	5.50(±0.00) ^{bc}	6.80(±0.00) ^b
	Effluent	7.60(±0.00) ^{bc}	8.20(±0.00) ^{bc}	8.90(±0.00) ^b
	Downstream	4.50(±0.00) ^b	5.50(±0.00) ^{bc}	6.80(±0.00) ^b
	Effluent water	6.56(±1.03) ^{bc}	7.30(±0.89) ^c	8.20(±0.70) ^b
May 2012	Upstream	4.83(±0.03) ^b	5.63(±0.03) ^{bc}	11.56(±0.66) ^b
	Effluent	10.20(±0.65) ^c	10.83(±0.63) ^{ba}	11.56(±0.66) ^{ba}
	Downstream	4.90(±0.09) ^{bc}	5.70(±0.03) ^{bc}	6.80(±0.00) ^b
	Effluent water	5.03(±0.09) ^{bc}	6.20(±0.40) ^b	7.40(±0.60) ^b

Where: treatments with different letters are significantly different ($P < 0.05$); \pm standard error of the mean (S.E.M.); $n=3$.

4.4.2. Biofilm biomass

Biofilm biomass was determined per cm² of the glass slide surface based on the concentration of extracted DNA, EPS and bacterial counts (**Table 4.2**). No correlation was found between biofilm biomass measured with assays described above. High variability of bacterial counts and DNA concentration suggested that these methods are not reliable. Therefore, concentration of EPS as glucose equivalents was mainly considered when comparing the biofilm biomass. Sampling date and location of biofilm collection point were found to significantly affect the amount of biofilm developed on glass slides. However, no statistically significant correlation was found between the DT values for PNP biodegradation, and the amount of biofilm biomass developed on the glass slides. Biofilms collected on glass slides appeared to be a good inoculum sources with higher biomass content than river water inocula.

Table 4.2: Inoculum size – biofilm biomass determined based on the DNA concentration, EPS concentration, and number of cultivable bacteria.

Date	Sampling site	DNA concentration (ng/L)	EPS as glucose equivalent (μg/L)	Bacterial counts (cfu/L)
November 2011	upstream	83.85 ^{abdf} (± 6.65)	250.69 ^a (± 3.80)	4.10x10 ^{7ace} (± 8.14 x10 ⁵)
	effluent	84.08 ^{abdf} (± 15.68)	73.18 ^b (± 0.63)	2.62x10 ^{6b} (± 1.77 x10 ⁵)
	downstream	73.61 ^{abdf} (± 5.05)	252.25 ^a (± 5.70)	6.90x10 ^{7c} (± 1.30x10 ⁷)
February 2012	upstream	15.89 ^{bd} (± 2.77)	227.05 ^c (± 2.21)	5.58x10 ^{6d} (± 1.43x10 ⁶)
	effluent biofilm	214.89 ^{efg} (± 41.38)	172.79 ^d (± 9.17)	3.93x10 ^{7e} (± 3.81x10 ⁶)
	downstream	10.23 ^d (± 0.53)	133.64 ^e (± 1.90)	1.14x10 ^{6f} (± 3.27x10 ⁴)
	effluent water	325.91 ^e (± 80.79)	NM	2.99x10 ^{5g} (± 1.28x10 ⁴)
May 2012	upstream	120.33 ^f (± 21.02)	667.75 ^f (± 1.26)	5.27x10 ^{6d} (± 2.72x10 ⁵)
	effluent biofilm	220.11 ^g (± 38.40)	261.16 ^a (± 0.95)	5.62x10 ^{6d} (± 1.26x10 ⁶)
	downstream	130.44 ^{acfg} (± 19.48)	635.19 ^g (± 0.63)	6.92x10 ^{7ac} (± 6.12x10 ⁶)
	effluent water	133.78 ^{acfg} (± 35.32)	NM	1.00x10 ^{6f} (± 1.39x10 ⁵)

Where: NM-not measured; treatments with different letters are significantly different (P<0.05); ± standard error of the mean (S.E.M.); n=3

4.4.3. Seasonal variation in physicochemical parameters

4.4.3.1. Water quality

River water and effluent quality characteristics (**Figure 4.3** and **4.4**) including COD, ammonia, nitrite, nitrate, DOC, pH, and light intensity and temperature were measured. COD, ammonia, DOC and temperature varied significantly between sampling sites, while nitrite, nitrate, pH and light were significantly different between sites and sampling dates. Significantly higher levels of ammonia were found in effluent and downstream river water in February 2012 than at the other sampling times. DOC was significantly higher in effluent compared to upstream and downstream samples collected on all sampling occasions. Also, the water temperature was significantly lower at upstream and downstream sites in comparison to the effluent discharge point.

Effluent flow and rainfall data were obtained from Severn Trent Water for the River Dene stretch with the Wellesbourne WWTP effluent discharge point. The data indicated that operation of Wellesbourne WWTP was according to standards for wastewater treatment with a consistent, good quality effluent, discharged to River Dene in November, February and May during the eight days of biofilm trap deployment.

4.4.3.2. Flow data

Measured water height, area and flow rate were used to calculate the total river discharge (**Table 4.3**) at each sampling site and for each time the biofilms were sampled. Higher level of river water and slower water flow was present at effluent sampling site in comparison to upstream and downstream sites. However, statistical significance was not determined since only single measurements were performed.

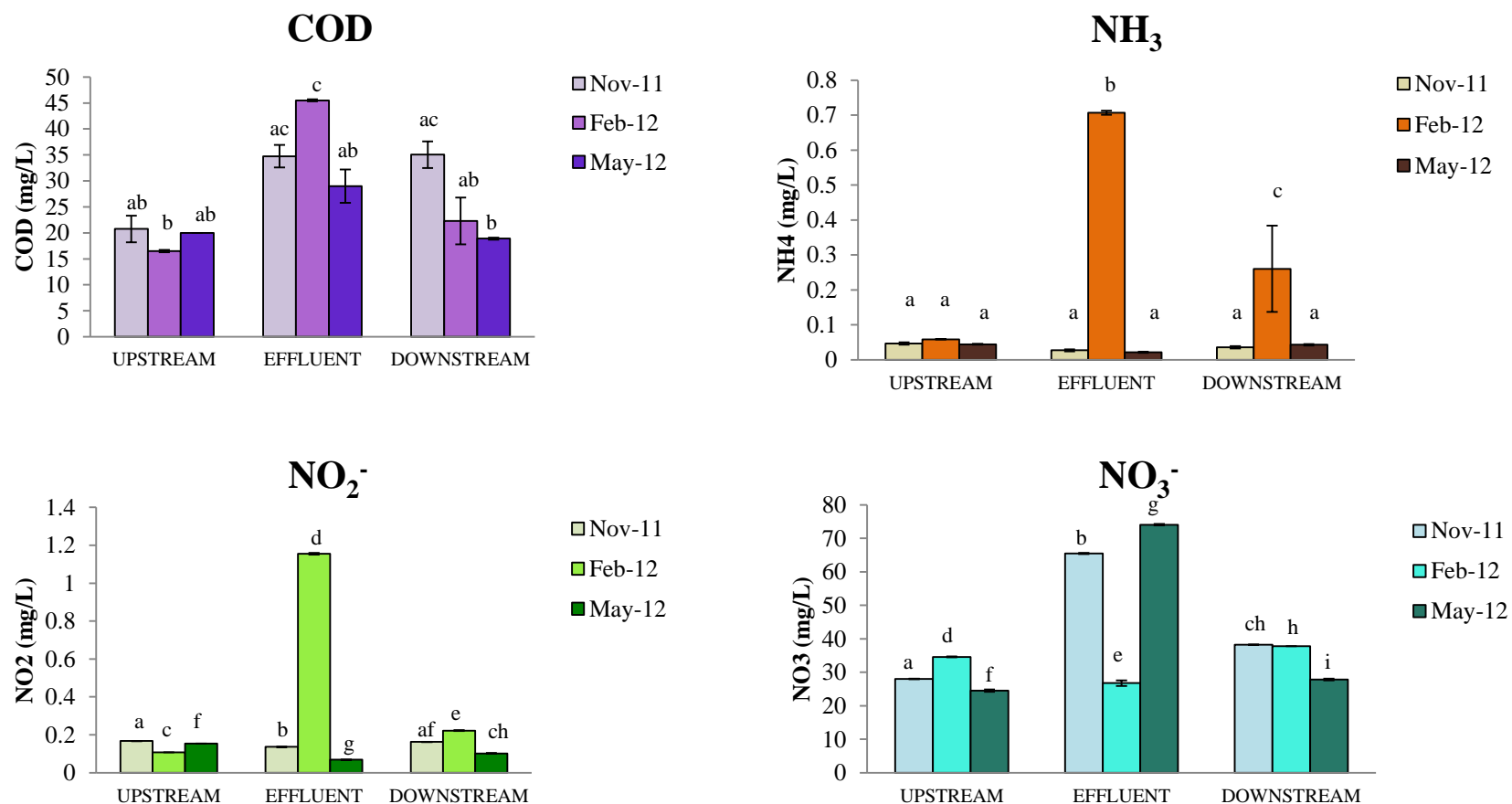


Figure 4.3: Measurements for COD, ammonia, nitrite and nitrate for river water at upstream, effluent and downstream site. Error bars showing \pm standard error of the mean (S.E.M.), n=3. Treatments with different letters are significantly different (P<0.05).

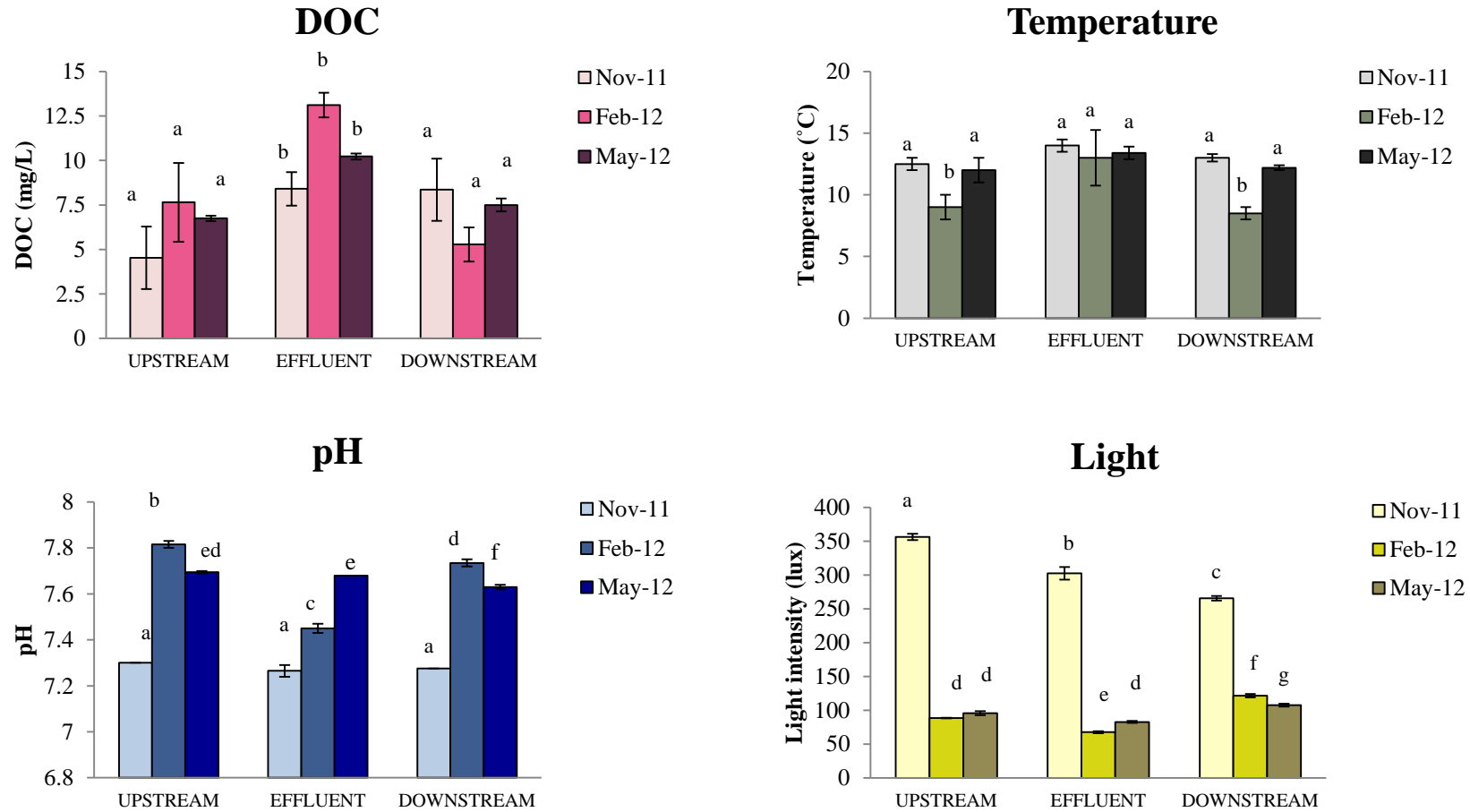


Figure 4.4: Measurements for DOC, temperature, pH and light at upstream, effluent and downstream site.

Error bars show \pm standard error of the mean (S.E.M.), $n=3$. Treatments with different letters are significantly different ($P<0.05$).

Table 4.3: River Dene water level, flow rate and water discharge.

Date	Sampling site	Measurements			
		Height (m)	Flow (m/sec) $\times 10^{-2}$	Area (m ²) $\times 10^{-2}$	Total discharge (m ³ /sec) $\times 10^{-2}$
November 2011	upstream	0.13	1.18	0.65	45.39
	effluent	0.21	0.32	1.05	23.06
	downstream	0.19	1.66	0.95	82.14
February 2012	upstream	0.15	2.97	1.49	28.26
	effluent	0.19	1.15	0.95	10.37
	downstream	0.21	3.25	1.63	29.94
May 2012	upstream	0.10	1.47	0.50	9.67
	effluent	0.17	0.25	0.85	11.55
	downstream	0.16	2.58	0.80	9.26

Where n=1

4.4.4. Biofilm and river water bacterial TRFLP profiles

4.4.4.1. NMDS analysis of TRFLP data

Bacterial TRFLP profiles of biofilms collected on different sampling dates and locations were compared and a comparison between river water and river biofilm bacterial profiles was also performed. ANOSIM revealed that the bacterial community structure of biofilms was significantly different between sampling dates (**Figure 4.5 a**) with specific profiles for each season and community shifts from T_0 to post-PNP degradation (T_{end}). Higher similarity (20%) was observed between TRFLP profiles for biofilms degrading PNP in February and May, and less than 20% similarity was found between TRFLP profiles from November biofilms and the biofilms collected in February and May after incubation with PNP. ANOSIM analysis of TRFLP profiles showed that location of biofilm sampling site had no effect on bacterial TRFLP profiles and upstream, downstream and effluent biofilms clustered together (**Figure 4.5 b**). Also, inoculum material did not affect community composition, since river water had similar bacterial profiles to river biofilm (**Figure 4.5 b**).

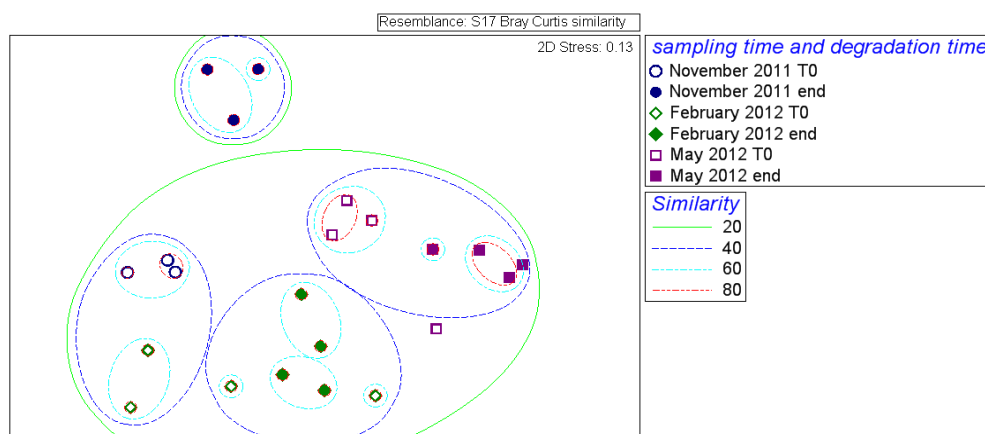
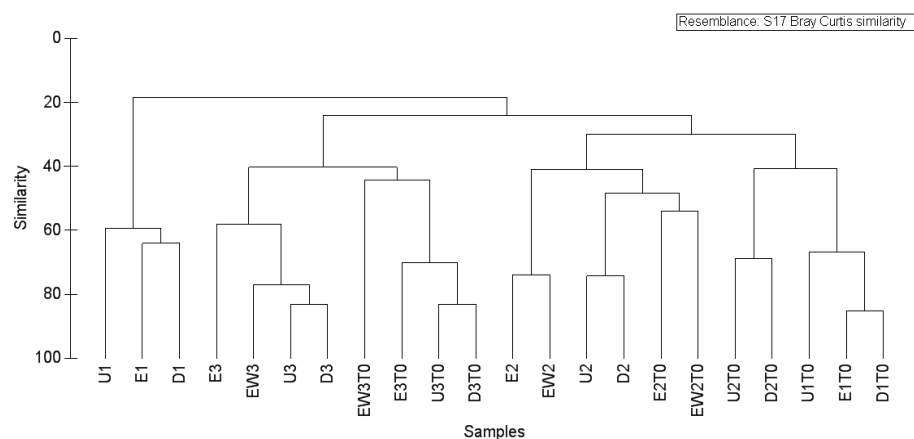
a**b**

Figure 4.5: Non-metric multidimensional scaling (NMDS) for bacterial TRFLP profiles, using Bray-Curtis similarity for the data set derived from amplification with bacterial primers 63F and 1087R- VIC fluorescently labelled and digested with *MspI* and *HhaI*. Stress: 0.13. Figure legend: a- NMDS plot showing effect of sampling date and PNP biodegradation on TRFLP profiles b- dendrogram showing effect of sampling date, location inoculum material and PNP biodegradation on bacterial profiles and their similarity, U- upstream biofilm, E- effluent biofilm, D- downstream biofilm, EW- effluent river water, 1- November 2011, 2- February 2012, 3- May 2012 T₀- freshly collected biofilm/river water, and T_{end}- biofilm/river water after incubation/complete PNP biodegradation.

4.4.4.2. Total number of Terminal Restriction Fragments (TRFs)

The total number of TRFs present in biofilms and river water bacterial profiles was calculated for each replicate (**Figure 4.6**). Sampling date had a significant effect on the number of TRFs present in biofilm and river water profiles with the highest number of TRFs observed for May biofilm TRFLP profiles (**Table 4.4**). Location affected the total number of TRFs in November biofilms after incubation and in February biofilms at T_0 . The least diverse bacterial profiles were obtained from November biofilms. No change in number of TRFs was recorded for November biofilms between T_0 and T_{end} . In February the number of TRFs increased significantly after PNP biodegradation, while May TRFLP profiles had lower number of TRFs after PNP biodegradation (**Figure 4.6**). Inoculum material had no impact on the number of TRFs.

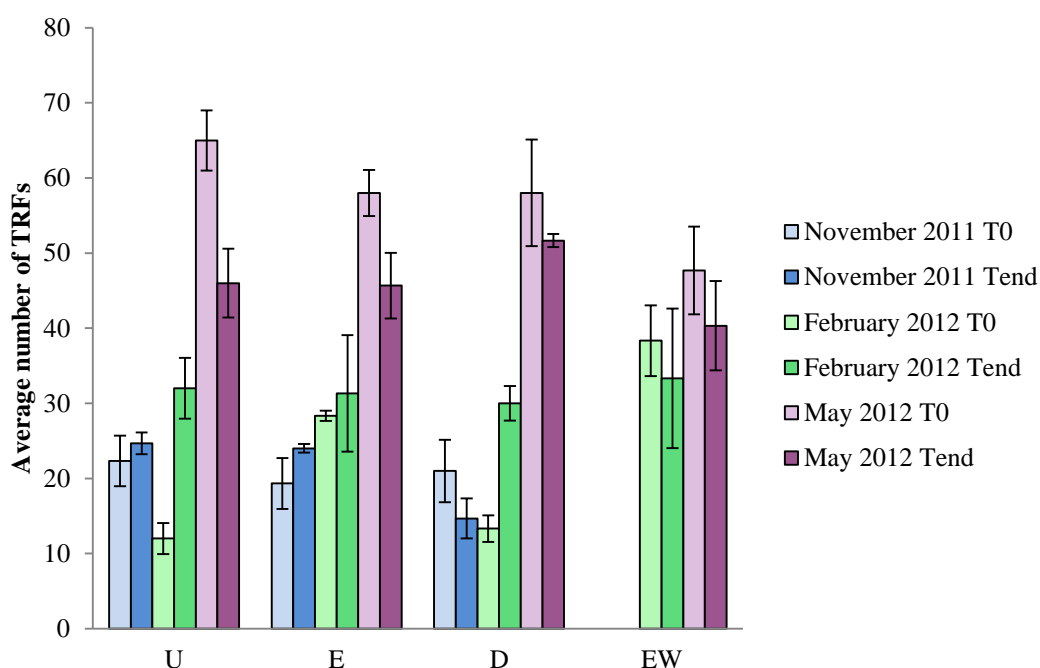


Figure 4.6: Total number of TRFs present in bacterial profiles from river water and biofilms collected in November 2011, February and May 2012. Figure legend: U- upstream biofilm; E- effluent biofilm; D- downstream biofilm; EW- effluent river water; T_0 - biofilm/river water before incubation; T_{end} - biofilm/river water after incubation/complete PNP biodegradation. TRFs obtained with primer 1087R-VIC fluorescently labelled. Error bars showing standard error of the mean (S.E.M), n=3.

Table 4.4: ANOVA for total number of TRFs present in biofilm and river water TRFLP profiles.

Variable		TRFLP profile	Average total number of TRFs	ANOVA		
Date and location	November 2011	upstream biofilm T ₀	22.3 (± 3.38)	0.849	0.229	<0.001
		effluent biofilm T ₀	19.3 (± 3.38)			
		downstream biofilm T ₀	21.0 (± 4.16)			
		upstream biofilm T _{end}	24.7 (± 1.45)	0.013		
		effluent biofilm T _{end}	24.0(± 0.58)			
		downstream biofilm T _{end}	14.7 (± 2.66)			
	February 2012	upstream biofilm T ₀	12.0 (± 2.08)	<0.001	0.060	
		effluent biofilm T ₀	28.3 (± 0.66)			
		downstream biofilm T ₀	13.3 (± 1.77)			
		upstream biofilm T _{end}	32.0 (± 4.04)	0.963		
		effluent biofilm T _{end}	31.3 (± 7.74)			
		downstream biofilm T _{end}	30.0 (± 2.31)			
May 2012	upstream biofilm T ₀	65.0 (± 4.00)	0.556	0.802		
	effluent biofilm T ₀	58.0 (± 3.05)				
	downstream biofilm T ₀	58.0(± 7.09)				
	upstream biofilm T _{end}	46.0(± 4.58)	0.479			
	effluent biofilm T _{end}	45.7(± 4.37)				
	downstream biofilm T _{end}	51.7(± 0.88)				
Date and material	November 2011	biofilm T ₀	19.3(± 3.38)	0.245	-	<0.001
		biofilm T _{end}	24.0(± 0.58)			
	February 2012	biofilm T ₀	28.3(± 0.66)	0.103	0.745	
		water T ₀	38.3(± 4.70)			
		biofilm T _{end}	31.3(± 7.74)	0.877		
		Water T _{end}	33.3(± 9.28)			
	May 2012	biofilm T ₀	58.0(± 3.05)	0.192	0.161	
		water T ₀	47.7(± 5.87)			
	biofilm T _{end}	45.7(± 4.37)	0.509			
	water T _{end}	40.3(± 5.92)				

Where: T₀- freshly collected biofilm/river water, T_{end}- biofilm/river water after complete PNP biodegradation; ± standard error of the mean (S.E.M.), n=3

4.4.5. Abundance of TRF 245 nt

Analysis with Primer 6 of the abundance of TRF 245 nt (**Figure 4.7 a and b**) showed differences between profiles of bacterial communities in T_0 and PNP degrading communities with increased abundance after complete PNP biodegradation (except for biofilms collected in November). The abundance of TRF 245 nt was different between biofilms sampled at different dates and significant differences were found between the location of biofilm sampling points in February (**Figure 4.7 a, Table 4.5**). The lowest abundance of TRF 245 nt was observed in November biofilms, with similar abundance in February and May biofilms. Also inoculum material affected the abundance of TRF 245 nt since effluent biofilm and water showed different abundance of this TRF (**Figure 4.7 b**). Higher abundance was observed in effluent river water (T_0) than in effluent biofilms in February, whereas May biofilms had initially higher abundance of TRF 245 nt than the river water (**Table 4.5**). Interestingly, the effluent river water collected in February had initially higher abundance of TRF 245 nt in comparison with river water post-PNP degradation. This is the opposite to the trend observed for the remaining biofilm and river water TRFLP profiles.

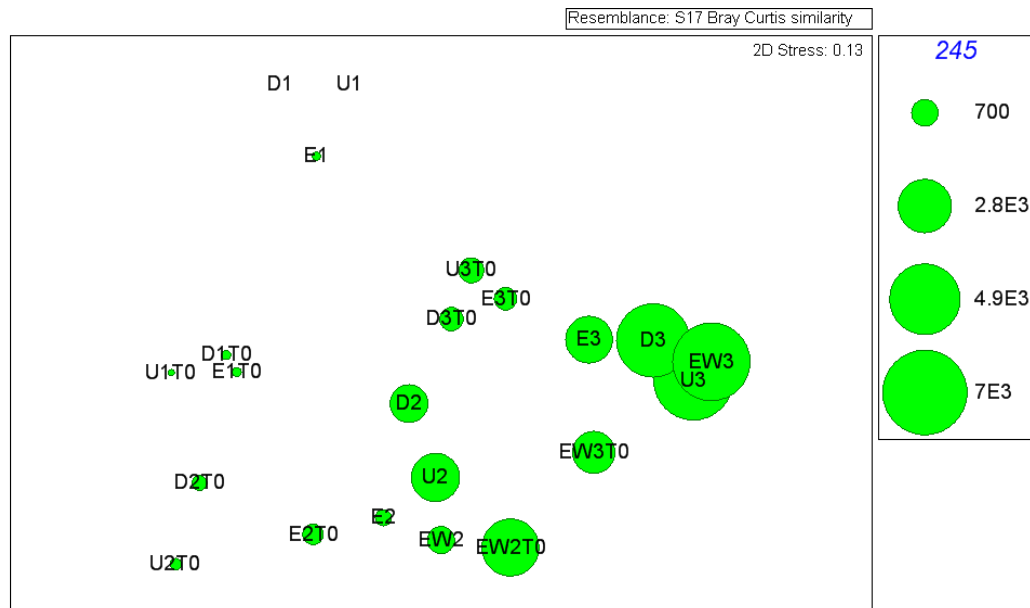
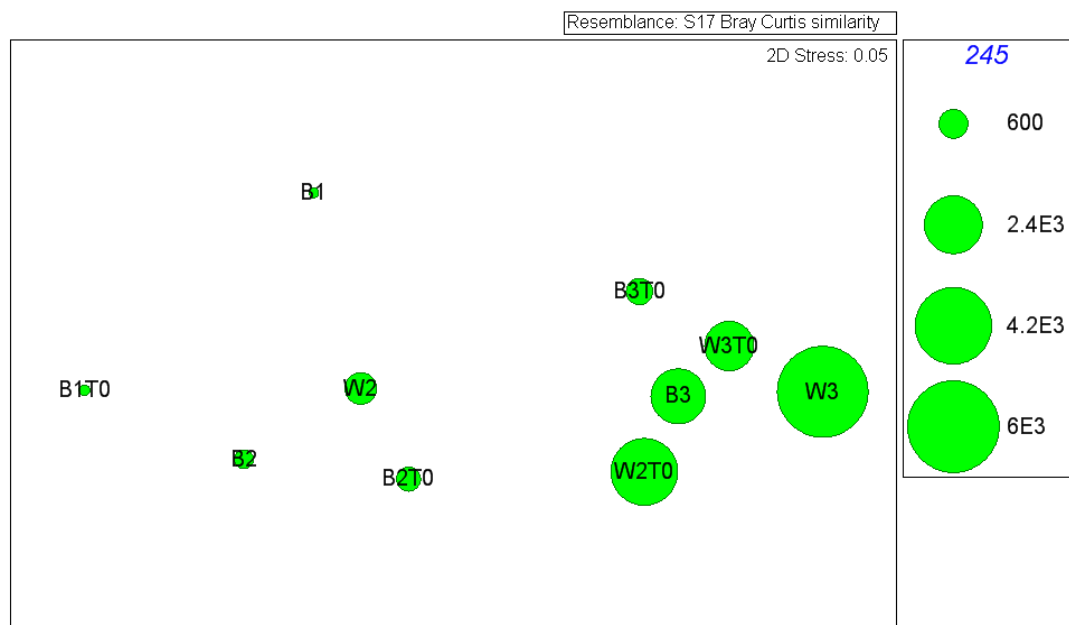
a**b**

Figure 4.7: A 2D bubble plot presenting the amount of TRF 245 nt (TRF identified as *Pseudomonas syringae*) using Bray-Curtis similarity. The area of green circles corresponds to the amount of TRF 245 nt present in environmental samples. Figure legend: **a**- effect of sampling date and location (stress: 0.13), **b**- effect of inoculum material (stress: 0.05), **1**- November 2011, **2**- February 2012, **3**- May 2012, **U**- biofilm upstream, **E**- effluent biofilm, **EW**- effluent river water **D**- downstream biofilm, **T₀**- freshly collected, letters without **T₀**- profiles after complete PNP biodegradation, **B**- river biofilm, **W**- river water.

Table 4.5: Relative abundance of TRF 245 nt in biofilm and river water TRFLP profiles.

Variable			TRFLP profile	Relative abundance of TRF 245 nt	ANOVA		
Date and location	November 2011	upstream biofilm T ₀	0.46(± 0.21)	0.379	0.151	<0.001	
		effluent biofilm T ₀	1.82(± 0.90)				
		downstream biofilm T ₀	1.54(± 1.92)				
		upstream biofilm T _{end}	0.00(± 0.00)	0.094			
		effluent biofilm T _{end}	0.91(± 0.52)				
		downstream biofilm T _{end}	0.00(± 0.00)				
	February 2012	upstream biofilm T ₀	3.83(± 0.31)	0.046	<0.001		
		effluent biofilm T ₀	4.78(± 0.31)				
		downstream biofilm T ₀	7.41(± 1.62)				
		upstream biofilm T _{end}	25.38(± 3.92)	0.003			
		effluent biofilm T _{end}	4.49(± 0.99)				
		downstream biofilm T _{end}	17.38(± 0.64)				
May 2012	upstream biofilm T ₀	3.72(± 0.24)	0.240	<0.001			
	effluent biofilm T ₀	3.18(± 0.27)					
	downstream biofilm T ₀	3.79(± 0.25)					
Date and material	November 2011	biofilm T ₀	1.82(± 0.89)	0.234	-	<0.001	
		biofilm T _{end}	0.91(± 0.52)				
	February 2012	biofilm T ₀	4.78(± 0.31)	<0.001	<0.001		
		water T ₀	20.65(± 1.66)				
		biofilm T _{end}	4.49(± 0.99)	0.114			
		water T _{end}	9.37(± 2.60)				
	May 2012	biofilm T ₀	3.18(± 0.27)	<0.001	<0.033		
		water T ₀	10.43(± 0.53)				
		biofilm T _{end}	14.07(± 3.45)	<0.001			
		water T _{end}	31.15(± 3.75)				

Where: T₀- freshly collected biofilm/river water, T_{end}- biofilm/river water after complete PNP biodegradation; ± standard error of the mean (S.E.M.); n=3.

4.4.6. Core and satellite taxa

Biodiversity has been defined as species richness and relative species abundance in space and time, where relative species abundance refers to the commonness or rarity of a species in relation to other species in the community (van der Gast *et al.*, 2011). Since the species abundance differs, an additional dimension of evenness can be used to define and discriminate ecological communities. A simple and effective method of illustrating differences in evenness and species richness in the local communities is the rank-abundance plot (Magurran, 2004). At the metacommunity level species can be divided into core and satellite groups, where the former group is comprised of species that are widely distributed and the latter group consists of rare species occurring in low abundance (van der Gast *et al.*, 2011).

4.4.6.1. Biofilm local community structure and abundance of *Pseudomonas syringae*

The rank-abundance plots were used to study the biofilm local community structure and the abundance of PNP-degrader *Pseudomonas syringae* (previously identified as TRF 245 nt) in communities of T_0 biofilms collected at different dates, from different sampling locations and to compare it with T_{end} biofilm local communities (**Appendix Figure 0.6 – 0.11**). The slopes of rank-abundance plots obtained were used to determine the evenness of structure of local communities, where slopes with values closer to zero indicate more even structure and those with increasingly negative values have a more dominant structure. ANOVA showed no differences between slopes of the rank-abundance plots for November biofilms between sampling locations and between T_0 and T_{end} which indicated that the biofilm community structure did not change after incubation with PNP. In February at T_0 effluent biofilm community structure was different from upstream and downstream biofilms. Significant changes in biofilm structure were observed between T_0 and T_{end} ($P=0.003$) upstream and downstream biofilms, with increased evenness of biofilm communities (decreased slopes of rank-abundance plots) after the biodegradation of PNP (**Table 4.6**). In May downstream biofilms had significantly different community structure at T_0 than upstream and effluent biofilms ($P=0.004$). After PNP

biodegradation increased evenness of community structure was observed for all biofilms. In addition a higher species richness was observed for all May biofilms in comparison with November and February biofilms. The rank-abundance plots also revealed that *P. syringae* was not present in one upstream local community in November at T_0 , while it was abundant in all T_0 local communities in February and May. Interestingly, the increased abundance of *P. syringae* was observed after incubation with PNP (T_{end}) in February and May but not in November. In November *P. syringae* was absent from all upstream and downstream local communities after incubation with PNP. No PNP biodegradation was observed for these biofilms (Figure 4.2).

Table 4.6: ANOVA test results comparing local biofilm communities from different sampling dates (November, February, May) sampling locations (upstream, effluent, downstream) and before (T_0) and after complete PNP biodegradation (T_{end}).

Date	Incubation time	Biofilm local community	Slope (b)
November 2011	T_0	upstream	-0.07 (± 0.01) ^a
		effluent	-0.06 (± 0.01) ^a
		downstream	-0.05 (± 0.01) ^a
	T_{end}	upstream	-0.06 (± 0.00) ^a
		effluent	-0.06 (± 0.00) ^a
		downstream	-0.08 (± 0.01) ^a
February 2012	T_0	upstream	-0.12 (± 0.03) ^a
		effluent	-0.05 (± 0.00) ^b
		downstream	-0.11 (± 0.02) ^a
	T_{end}	upstream	-0.04 (± 0.01) ^b
		effluent	-0.04 (± 0.01) ^b
		downstream	-0.04 (± 0.00) ^b
May 2012	T_0	upstream	-0.07 (± 0.02) ^{ab}
		effluent	-0.05 (± 0.00) ^{bd}
		downstream	-0.11 (± 0.02) ^c
	T_{end}	upstream	-0.04 (± 0.01) ^d
		effluent	-0.04 (± 0.01) ^d
		downstream	-0.04 (± 0.00) ^d

Where: treatments with different letters are significantly different ($P < 0.05$); \pm standard error of the mean (S.E.M.); $n=3$

4.4.6.2. Biofilm metacommunity structure, distribution and persistence of *Pseudomonas syringae*

Rank abundance plots were performed for metacommunities to determine the changes in biofilm community structure between T_0 and T_{end} biofilms (Appendix Figure 0.12). Based on the slopes of rank-abundance plots (Appendix Table 0.6) it

was shown that at the metacommunity level February biofilm community structure became more even after PNP biodegradation while November and May biofilms shifted to less even communities. Higher species richness was observed in May than in November and February biofilms. *P. syringae* was more abundant in February and May but less abundant in November metacommunity. Also the abundance of *P. syringae* increased after PNP biodegradation in February and May, but decreased in November.

After dispersion was plotted against the persistence of taxa for biofilm metacommunities, Poisson distributions were fitted (**Appendix Figure 0.13-0.15**). The taxa which fell below the 2.5% confidence limit line were identified as randomly distributed satellite species. Taxa above the 2.5% confidence limit line were non-randomly distributed core species. Overall, a higher number of satellite taxa was observed at T_{end} than at T_0 in November, February and May metacommunity. The number of core taxa increased from T_0 to T_{end} in February but decreased in November and May biofilms at T_{end} . After partitioning taxa into core and satellite species the rank-abundance plots were performed for all metacommunities (**Figure 4.8**). It appeared that *P. syringae* was a core taxon at T_0 in the November and February biofilm metacommunities but was identified as a satellite taxon in the May T_0 metacommunity. After the biodegradation of PNP (T_{end}) *P. syringae* became more persistent and remained a core taxon in February but shifted from satellite into a core taxon in May. In November the persistence of *P. syringae* decreased after incubation with PNP and *P. syringae* shifted from core to a satellite taxon. Analysis of slopes of rank-abundance plots (**Table 4.7**) revealed significant differences between the T_0 and T_{end} metacommunities and shifts in the November and February metacommunities were observed to more even community structure after PNP biodegradation, but in May the biofilm metacommunity became more dominated (less even). In addition, sampling dates were found to have a significant impact on biofilm metacommunity since different biofilm community structure was observed in May than in November and February at T_0 (**Table 4.7**).

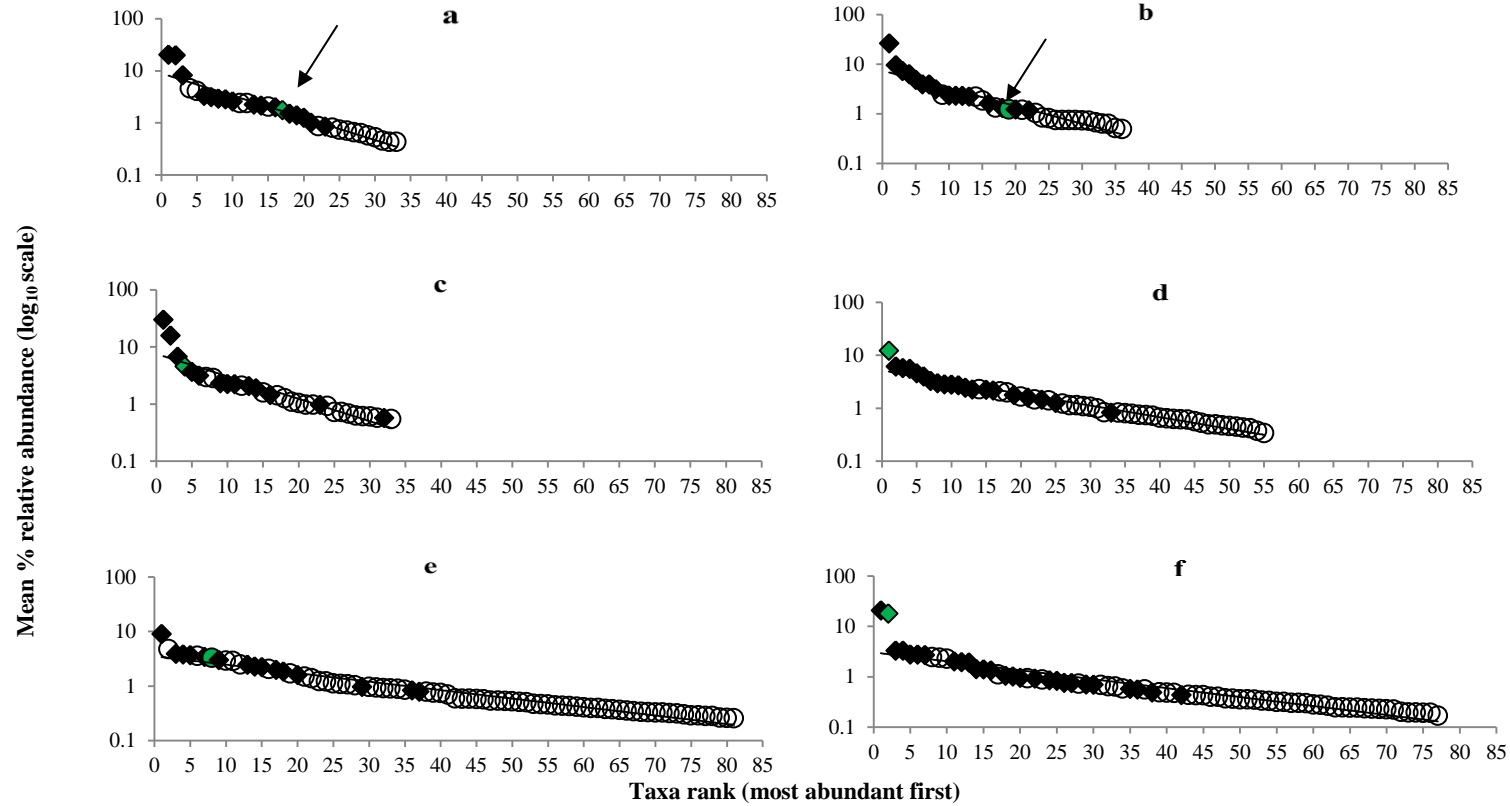


Figure 4.8: Rank-abundance plots with partitioning into core and satellite species.

Where: **a, b**- November T_0 and T_{end} , **c, d**- February T_0 and T_{end} , **e, f**- May T_0 and T_{end} , T_0 - freshly collected biofilms, T_{end} – biofilms after complete PNP biodegradation. The relative rank positions of core group (closed diamonds), satellite group (open circles) and *Pseudomonas syringae* (highlighted in green) are given. Each plot has been fitted with a slope.

Table 4.7: Comparison of slopes of metacommunity rank-abundance plots with core and satellite taxa.

Rank-abundance plot	F-value	Slope (b)	P-value	r²
November T ₀	F _{1,31} =315.78	-0.0426 ^a	<0.0001	0.91
November T _{end}	F _{1,34} =266.61	-0.0356 ^b	<0.0001	0.89
February T ₀	F _{1,31} = 165.96	-0.0386 ^{ac}	<0.0001	0.84
February T _{end}	F _{1,53} =1176.32	-0.0221 ^d	<0.0001	0.96
May T ₀	F _{1,79} =1362.73	-0.0155 ^e	<0.0001	0.95
May T _{end}	F _{1,75} =452.11	-0.0177 ^f	<0.0001	0.86

Treatments with different letters are significantly different (P<0.05); n=3.

4.4.7. Factors affecting biofilm bacterial community structure

Using Primer 6, TRFLP profiles were matched with environmental variables; river flow and discharge area, temperature, light, and river water quality. ANOSIM revealed that river water height, discharge area, temperature and nitrite concentration were mainly responsible for grouping T₀ biofilm bacterial TRFLP profiles from November 2011 and February 2012 separately from profiles obtained in May 2012 (**Figure 4.9**). Except for nitrite levels in upstream biofilms collected in May 2012 (marked on **Figure 4.9 d**) which were not significantly different from biofilms in November 2011 and February 2012. Environmental variables which best match the multivariate pattern of T₀ bacterial TRFLP profiles were selected for BEST analysis and the correlation between those variables and bacterial TRFLP profiles was determined. Statistically significant relationship was found between T₀ bacterial TRFLP profiles and river discharge area (r (25)=0.444, P<0.05), while no correlation was observed for temperature, nitrite concentration and river water height.

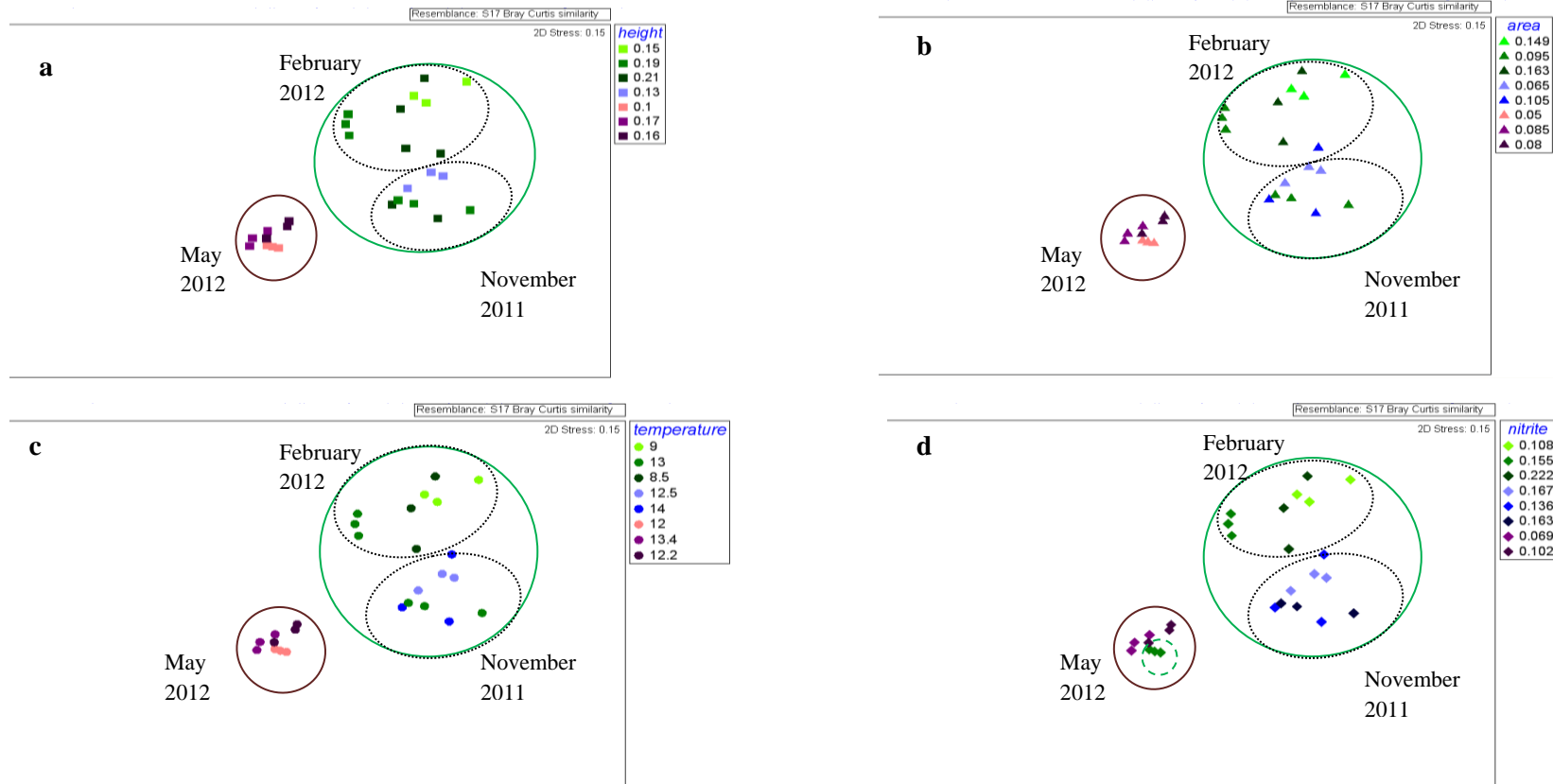


Figure 4.9: Non-metric-multidimensional scaling plots showing environmental factors affecting biofilm bacterial community structure at T_0 . Figure legend: **a-** water level (height) (m), **b-** section discharge area (m²), **c-** temperature (°C) and **d-** nitrite concentration (mg/L), **green circle-** no difference, **pink circle** – significant difference.

4.4.8. Quantitative PCR analysis of functional genes

4.4.8.1. Copy number of *pnpA*

ANOVA for the copy number of *pnpA* (**Figure 4.10**) showed significant differences in the copy number of *pnpA* between the sampling dates with higher *pnpA* copy number detected in inocula collected in February 2012. There were no differences in the copy number of *pnpA* at T_0 between biofilms collected from different sampling points in November, but significant differences were found between T_0 biofilms and effluent river water collected in May. Significantly higher *pnpA* copy number was observed in biofilms that degraded PNP and those that did not at T_{end} . Also, the *pnpA* copy number was significantly different before and after PNP biodegradation in February and May biofilms. An enrichment of copy number of *pnpA* was observed in biofilms degrading PNP. Due to the presence of false positives (as discussed in Chapter 2) copy number for *pnpA* do not present the actual abundance of PNP-degraders in the samples.

4.4.8.2. Copy number of *mar*

ANOVA for the copy number of *mar* (**Figure 4.11**) revealed significant differences (except for biofilms in May 2012) in the *mar* copy number, with a higher copy numbers detected in inocula collected in February 2012. Copy number was affected by the time of biofilm collection. Inoculum material had no impact on *mar* copy number, since no significant differences were found between river water from effluent outfall and effluent biofilm. In November, higher *mar* copy number was detected in effluent biofilm at T_{end} . Sampling site location affected the copy numbers of *mar* gene and higher copy number of that gene were present in T_0 upstream and downstream biofilms collected in February. Significantly different copy numbers of *mar* were also detected before and after PNP biodegradation in February biofilms, collected from upstream and downstream site, but no differences were found in May biofilms. However, copy number for *mar* does not represent PNP-degraders only (as discussed in Chapter 2)

4.4.8.3. Copy number of 16S rRNA gene

In November, copy number of the 16S rRNA gene (**Figure 4.12**) at T_0 was higher in upstream and downstream biofilm than in the effluent biofilm. At T_{end} copy number was higher in upstream and effluent biofilms than downstream biofilms at the end of incubation. A significantly lower copy number was found in February effluent river water than in effluent biofilm at T_0 . After complete PNP biodegradation, a higher copy number of 16S rRNA genes was detected in effluent river water but not in the biofilms. In May, differences were observed between the copy number of 16S rRNA gene in different biofilm inocula at T_0 and a higher copy number of 16S rRNA gene was detected in downstream biofilm than in the other biofilms. After complete PNP biodegradation lower 16S rRNA gene copy number were present in all biofilms and effluent river water than at T_0 . It is noteworthy that the counts are very low for 16S rRNA gene (possibly due to the long gene fragment amplified) and probably do not represent the actual abundance of bacteria in the samples.

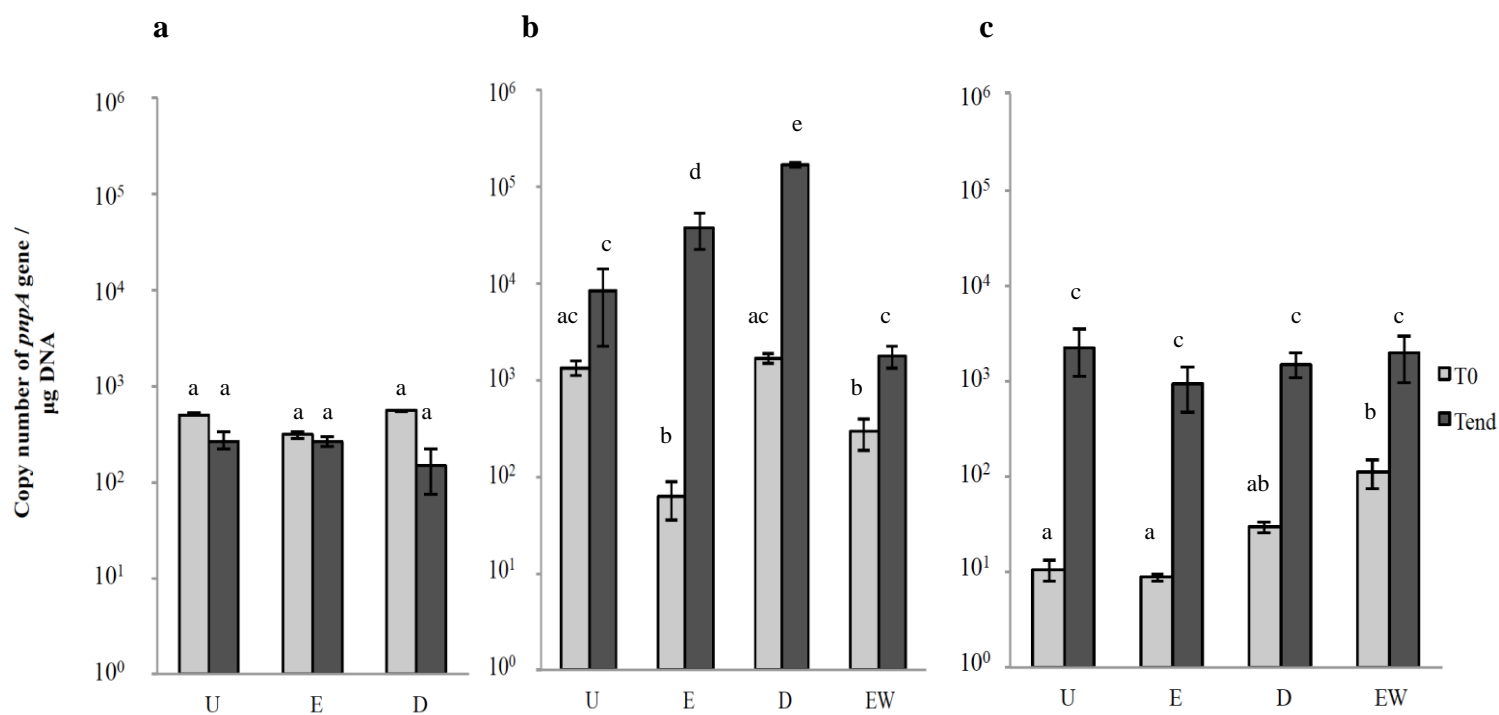


Figure 4.10: Copy number of *pnpA* gene. Figure legend: **a**- November 2011, **b**- February 2012, **c**- May 2012; **U**- upstream biofilm, **E**-effluent biofilm, **D**- downstream biofilm, **EW**-effluent water; treatments with different letters are significantly different ($P < 0.05$); error bars showing \pm standard error of the mean (S.E.M.), $n=3$.

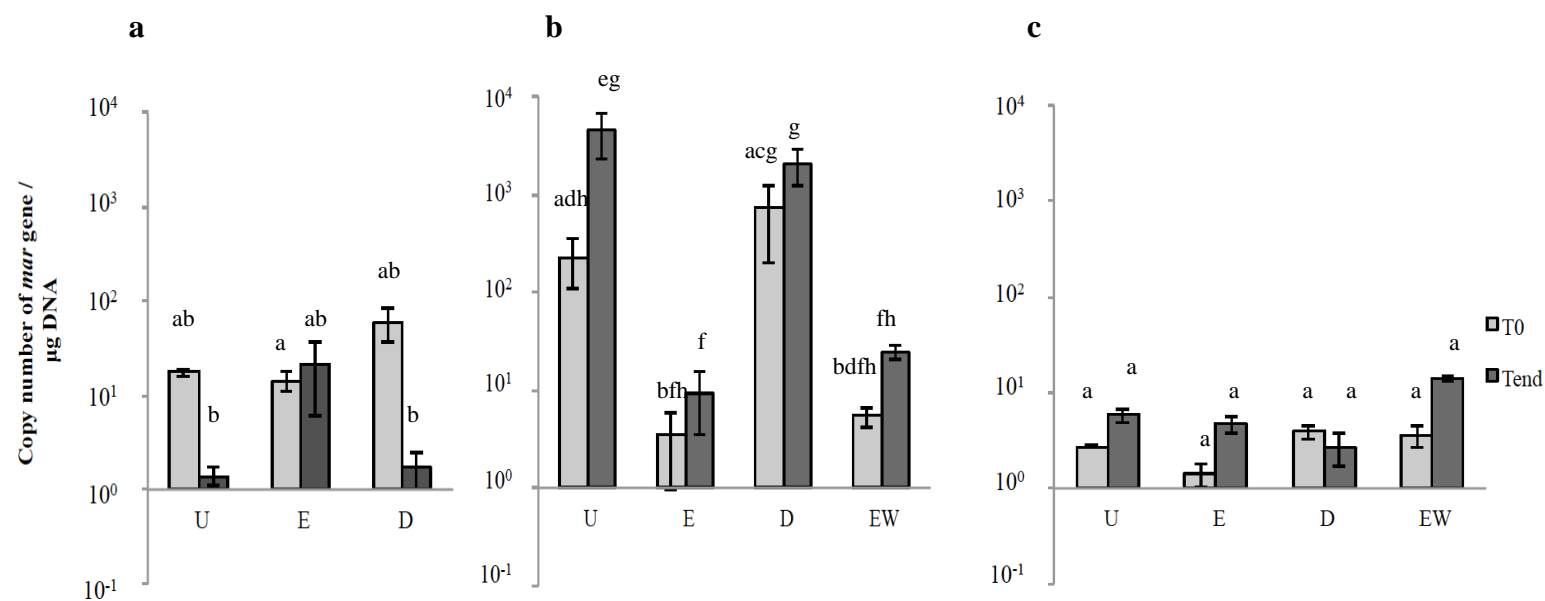


Figure 4.11: Copy number of *mar* gene. Figure legend: **a**- November 2011, **b**- February 2012, **c**- May 2012; **U**- upstream biofilm, **E**-effluent biofilm, **D**- downstream biofilm, **EW**-effluent water; treatments with different letters are significantly different ($P<0.05$); error bars showing \pm standard error of the mean (S.E.M.), $n=3$

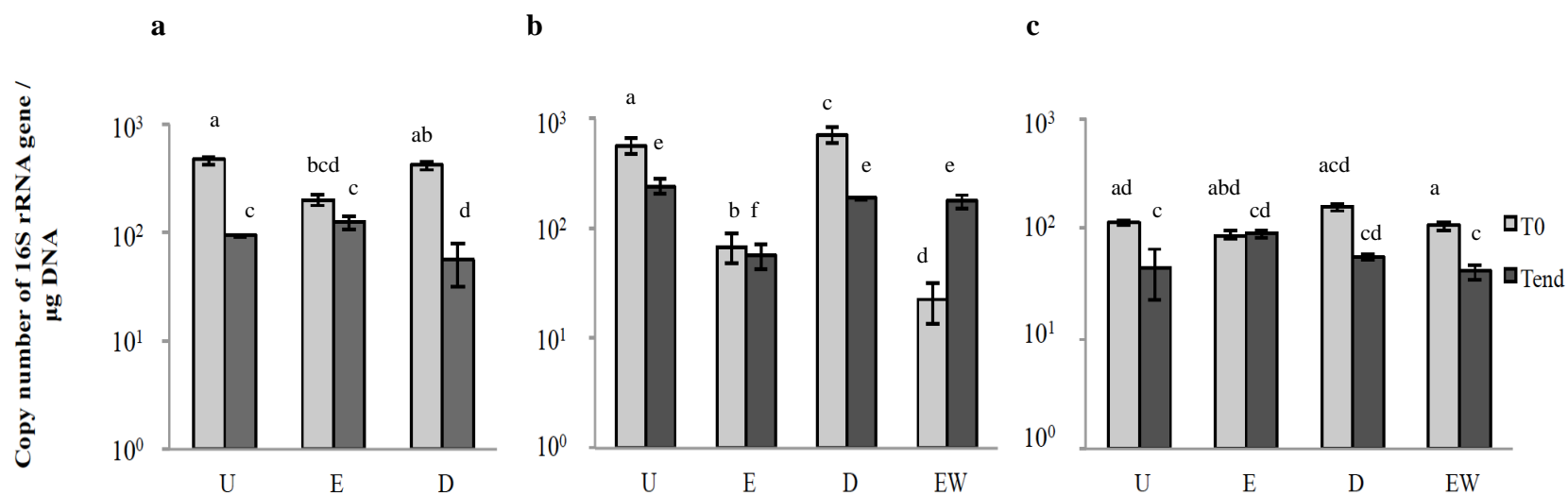


Figure 4.12: Copy number of 16S rRNA gene. Figure legend: **a**- November 2011, **b**- February 2012, **c**- May 2012; **U**- upstream biofilm, **E**-effluent biofilm, **D**- downstream biofilm, **EW**-effluent water; treatments with different letters are significantly different ($P<0.05$); error bars showing \pm standard error of the mean (S.E.M.), $n=3$

4.5. Discussion

4.5.1. Effect of sampling date on the biodegradation of PNP and microbial community composition

The major aim of this study was to investigate the reproducibility of PNP biodegradation using river biofilms as source of inoculum. ANOVA for DT values (**Table 4.1**) for PNP biodegradation with biofilms collected in November, February and May indicated that the sampling date impacted on the river biofilms biodegradation potential. Although, no correlation was observed between the biofilm biomass, i.e. DNA concentration, EPS concentration and number of cultivable bacteria, and DT values, significant differences were found between the amount of biomass in biofilms and river water collected at different times of the year. Overall, higher biodegradability was observed for biofilms collected in winter (February) than in spring (May) with low potential to degrade PNP with biofilms collected in the autumn (November). However, differences in PNP biodegradation in November were difficult to determine, because upstream and downstream biofilms failed to degrade PNP. In a study of seasonal variability of biodegradation of mixed amino acids, an anionic surfactant, detergent builder and herbicide, conducted by Palmisano *et al.* (1991), lower first-order rate constants for chemical mineralization were obtained with winter biofilms in comparison with summer biofilm samples.

The results also indicated that some environmental factors i.e. river discharge area could have affected the T_0 biofilm bacterial community structure (**Figure 4.9**), although there was no correlation between these data and the PNP biodegradation test results. Nevertheless, temperature is one of the key environmental variables affecting microbial biomass (LaPara *et al.*, 2000) and chemicals mineralization (Palmisano *et al.*, 1991). While water level affects the concentration of nutrients and chemicals which are important for bacterial biomass production (Carvalho *et al.*, 2003) and could affect stream biodegradation processes (Yuan *et al.*, 2004)

In the current project significant differences were found for water quality between the sampling dates and sites, which might have impacted the activities of biofilm bacteria. However consistent dilution of effluent discharged to the River Dene (1:10)

was observed at effluent discharge point regardless of the sampling date. This study also revealed differences in temperature and light intensity between November, February and May. It is well known that environmental variations and anthropogenic sources of chemicals affect the bacterial communities present in the environment (Yannarell and Triplett, 2005; Rubin and Leff, 2007). In water ecosystems, environmental conditions, such as temperature and nutrients, vary considerably with the season (Chénier *et al.*, 2003). Therefore, seasonal variation of biofilm biomass could have been caused by changes in environmental conditions and water quality between the different sampling dates in November, February and May.

The present study also revealed that biofilm and river water TRFLP bacterial profiles (**Figure 4.5 a and b**) were significantly affected by the sampling date. The abundance of TRF 245 nt also varied (**Figure 4.7 a** and **Table 4.5**) although it was identified as a core taxon in studied TRFLP profiles. Analysis of local (section **4.4.6.1**) and metacommunities of biofilms (section **4.4.6.2**) also demonstrated that sampling date affected biofilm community structure, and persistence and distribution of the PNP-degrader *P. syringae*. These results correlate with PNP biodegradation results, where successful PNP biodegradation was also affected by season. Additionally, the copy numbers of PNP functional markers for PNP biodegradation, *pnpA* and *mar* showed significant differences between sampling dates (**Figure 4.10** and **4.11**). This might indicate seasonal differences in the abundance of PNP-degraders which could result in the variable biodegradation potential of biofilm inocula sampled at different dates. Brümmer *et al.*, (2000) studied the biofilm community structure in polluted rivers over a complete annual cycle, and reported clear seasonal peaks of abundance among major phylogenetic groups of bacteria. The composition of biofilms may affect their potential for chemical biodegradation. Interestingly, Böckelmann *et al.* (2000) described a reduction of metabolic activity of microbial communities of aggregates of phototrophic and heterotrophic microorganisms in the Elbe River, from summer (August) to winter (December) for both, free-living and surface associated bacterial populations. Changes in biofilm community structure and function may impact on biofilm potential for chemical biodegradation. According to Chénier *et al.* (2003) biofilm from river water collected in autumn showed the highest hexadecane mineralization rate, and the summer biofilm showed the lowest activity, while winter and spring biofilms were

intermediate. It was also reported that the biofilm community response to nutrients varied with season, and community composition was an important variable in response to environmental stress (Chénier *et al.*, 2003).

All results from this section supported the initial hypothesis that the sampling date affects the bacterial community composition present in river biofilms and water samples, and therefore, impacts on the DT-values derived from PNP biodegradation tests.

4.5.2. Effect of sampling site location on the biodegradation of PNP and microbial community composition

The location of the biofilm sampling point affected the biodegradation of PNP but had no impact on the bacterial community structure within biofilms. The PNP biodegradation and calculated DT values (**Table 4.1**) showed a significant effect of biofilm sampling point location on their biodegradation potential. The amount of biofilms developed on glass slides (based on EPS concentration) was also significantly different at each location (**Table 4.2**). The EPS concentration was chosen as the most appropriate measure of biofilm biomass since it is typically used for biofilm characterization (Schaule *et al.*, 2000; Bruckner *et al.*, 2008). However no significant correlation was found between the inoculum size i.e. biomass content, and the biodegradation of PNP. Although, effluent biofilms collected at different dates were efficient in the biodegradation of PNP, faster PNP biodegradation was observed for river water collected in May at the effluent discharge point and biofilms collected in February and May from upstream and downstream site of that point. These results corroborated findings of Writer *et al.* (2011) who reported that mineralization of 17 β -estradiol was not significantly different in biofilm and sediment matrices collected upstream and downstream from a WWTP.

Bacterial community composition (**Figure 4.5 a and b**) was not different between sites but the abundance of TRF 245 nt was different between February biofilms collected from effluent discharge point, upstream and downstream of that point (**Figure 4.7 b and Table 4.5**). Rank-abundance plots also revealed differences in biofilm community structure and the abundance of PNP-degrader, *P. syringae*

between sampling locations in February and May (**Table 4.6**). These results correlate with PNP biodegradation data, since it was observed that only these biofilms which degraded PNP (**Figure 4.2**) had a high abundance of *P. syringae*, and it was non-randomly distributed (core taxon) in these biofilms (**Figure 4.8**). The copy numbers of PNP functional markers also differed significantly after PNP biodegradation between the biofilms collected from different sites (**Figure 4.10 b**) and differences were found between functional gene copy numbers at T₀ (**Figure 4.10** and **4.11**). Results of the current study confirmed the initial hypothesis that the location of biofilm sampling site affects their potential for PNP biodegradation. However, the lack of impact of location on the biofilm community structure proved that the hypothesis that biofilm community structure is affected by sampling site was incorrect.

4.5.3. Effect of inoculum material on the biodegradation of PNP and microbial community composition

The effect of inoculum material (water or biofilm) on the biodegradation of PNP and inoculum community composition was determined based on the comparison of results obtained for effluent river water and effluent biofilms. ANOVA for DT-values calculated for PNP biodegradation with inocula collected in May (**Table 4.1**) indicated that inoculum material significantly affects the biodegradation of PNP. The biomass content was significantly higher in effluent biofilms than in effluent river water (**Table 4.2**). An influence of inoculum material was previously described by Mezzanotte *et al.* (2005), who used three activated sludges from industrial and two municipal WWTPs as inoculum for the biodegradation of plastic materials. It was reported that different maximum biodegradation percent was obtained with studied inocula.

Results from current experiment showed no effect of inoculum material on bacterial community structure since TRFLP profiles found in effluent biofilms were similar to river water profiles (**Figure 4.5 b**). However, the abundance of TRF 245 nt was higher in effluent river water than in the biofilm inoculum (**Figure 4.7 b** and **Table 4.6**). Based on the results from Chapter 3 and the current section, it can be deduced

that microbial communities found in river water also contribute to biofilm formation on surfaces like glass slides, but different communities are present in the surface layer of river sediment.

Additionally, QPCR for functional genes *pnpA* and *mar* revealed higher initial copy numbers of both markers in effluent river water than in the biofilm at T_0 . Once again, effluent river water proved to be a good inoculum material. Results described in this section confirmed the hypothesis that inoculum material impacts on the outcome of PNP biodegradation tests but the initial hypothesis that inoculum material affects microbial community structure was rejected.

This study revealed a relationship between sampling date, sampling location and inoculum composition and how these affect the inoculum's potential to degrade PNP. This has important consequences for biodegradation test results since it was shown that the biodegradation of PNP was affected by the inoculum sampling date and that changes in environmental variables may also affect the inoculum biodegradation potential. It was also demonstrated that river effluent biofilms represent a good quality inoculum for biodegradation tests and application of such inocula gave the possibility to increase the amount of bacterial biomass in biodegradation test systems. Although, inoculum characterization (quantity and quality) was applied, the prediction of biodegradation potential of inocula using developed microbial ecology approaches is still challenging. However, good correlation between the abundance of TRF 245 nt, specific to PNP-degrading bacterial populations, and the complete PNP biodegradation indicates a certain degree of probability based on which a prediction of biodegradation potential could be made.

It is therefore important to better understand the inocula used in biodegradation studies to minimise false positives and to better inform the chemical management decision making. Knowledge and development of reliable methods which could predict the biodegradation potential of test inocula would be essential for chemical risk assessment in natural environment.

4.6. Conclusions

Results in this chapter lead to the following conclusions:

1. Biodegradability potential of river water biofilms was affected by sampling date and location.
2. Sampling date affected the bacterial community structure within biofilms, possibly due to the changes in the river discharge area.
3. Although river water had similar bacterial community structure to river biofilms inoculum material had an impact on the biodegradation of PNP.
4. Copy numbers of *pnpA* and *mar* functional markers were affected by sampling date and the inoculum material. An enrichment of copy number of both functional genes was observed in river water and biofilms degrading PNP.
5. TRF 245 nt was identified as a core or a satellite TRF (depending on sampling date) which was present in biofilm and river water TRFLP profiles and it seems to be a good indicator of inoculum potential for PNP biodegradation. However an increase in the abundance of TRF 245 nt was not always observed after complete PNP biodegradation suggesting that perhaps other bacterial populations were also involved in PNP biodegradation.
6. The biodegradation of PNP lead to increased evenness of biofilm local community and increased abundance of *P. syringae*. At the metacommunity level, PNP biodegradation resulted in an increased persistence of *P. syringae* and changes in its distribution from a satellite to core taxon.
7. Effluent biofilms appeared to be good quality inoculum improving the reproducibility of biodegradation tests.
8. Application of river water biofilms enabled concentration of bacterial biomass and increased bacterial density in biodegradation test systems.
9. River biofilms represent bacterial populations which are abundant in flowing river water.
10. OECD biodegradation test guidelines need to account for inocula to better understand their variability, potential to biodegradation of chemicals, and how to use that knowledge for better chemical management.

11. Use of biofilms may reduce the discrepancy between the lack of inoculum consideration in OECD test guidelines and the role of microbial inocula in the biodegradation of chemicals.

Chapter 5

Threshold PNP concentration for the biodegradation of PNP

5.1. Introduction

Production and release of chemicals into the environment requires an assessment of their potential effects on natural ecosystems. In order to predict chemical persistence and ecotoxicological effects the Organization for Economic Cooperation and Development (OECD) has established a series of laboratory tests. Although OECD tests were designed to provide consistent results and they are standardized methods for comparative chemical assessment and prioritization, they are conducted under environmentally unrealistic conditions and extrapolation of biodegradation data obtained in the laboratory to conditions in the real environment makes risk assessment challenging.

One of the underlying reasons for this are the chemical concentrations typically used in OECD laboratory biodegradation studies that are unrealistic and significantly higher than found in the natural environment. For instance, *para*-nitrophenol (PNP) is present at low concentrations in natural environments and PNP concentration may range from ng/L in the air to µg/L in rivers or mg/kg in street dust (**Table 5.1**).

Table 5.1: Concentrations of PNP measured in the environment.

Environment	Geographical location	PNP concentration measured	References
air	Europe (German Alps, Fichtelgebirge, Germany; Mount Brocken, Germany; Great Dun Fell summit, United Kingdom)	1.2-360 ng/L	Herterich and Herrmann, 1990; Lüttke <i>et al.</i> , 1997
air	Japan	1-71 ng/L	Japan Environment Agency, 1995
street dust	Japan	42 mg/kg	Nojima <i>et al.</i> , 1983
rain water and snow	Germany and USA	<0.5-19 µg/L	BUA, 1992
river	German and Dutch parts of River Rhine	1 µg/L	BUA, 1992
river	Malaysian Klang river basin	<0.1-18.8 µg/L	Tan and Chong, 1993

Extrapolation to real world situation using results where high concentrations are applied may not reflect the processes which occur at low chemical concentration in the environment (Xu *et al.*, 2009). Adapted microbial communities may fail to degrade test compounds when they are present at low concentration since the extent of adaptation may increase with chemical concentration (Berg and Nyholm, 1996). Several authors have found concentration thresholds for biodegradation of chemicals, for example linear alcohol ethoxylates (LAE) and LAE sulfates in estuarine water (Vashon and Schwab, 1982), and 2,4-dichlorophenoxyacetate in stream water (Boethling and Alexander, 1979), and Battersby (1990) demonstrated that these threshold concentrations could be of the same order of magnitude (µg/L) as their environmental concentrations. Very low chemical concentrations found in natural environments affect the rate of entry of substrate into the cell (Battersby, 1990), and since many microbial transporters and catabolic enzymes are regulated and are synthesized in response to the presence of a certain concentration of their substrate (Harms and Bosma, 1997) the induction of enzymes which are involved in the biodegradation pathways of test compounds might require higher concentration of chemical (Hanne *et al.*, 1993).

Pandey and Jain (2002) suggested that bacterial chemotaxis, movement under the influence of chemical gradient, may impact on the efficiency of biodegradation processes. Toxic levels of chemicals will prevent biodegradation-competent bacterial cells from degrading a chemical while low concentrations will attract cells of chemical degrading bacteria and enable them to find the optimum conditions for their growth (Pandey and Jain, 2002). For instance, chemotaxis of *Ralstonia* sp. strain SJ98 was reported by Samanta *et al.* (2000) towards different nitroaromatic compounds, followed by their biodegradation. Therefore, affinity of bacterial cells to chemicals (carbon source) affects the chemical degradation. Joergensen and Degn (1987) studied the growth rate of *Methylococcus capsulatus* and its affinity to methane. Increased affinity of bacterial cells to methane increased the bacterial growth rate and resulted in faster methane utilization.

Often, chemicals at low concentrations do not serve as primary substrates in the environment where a variety of compounds are present (Ramakrishnan *et al.*, 2011). Hence, the primary contaminant may be co-metabolized in the presence of other potential carbon sources in river water or soil, which results in lower biodegradation rates of tested compound (Wang *et al.*, 1984; Novic and Alexander, 1985) compared to growth-linked biodegradation kinetics typical in the presence of higher chemical concentration (Vercé *et al.*, 2001; Bai *et al.*, 2007). Differences in biodegradation kinetics were also reported for high and low chemical concentrations by Battersby (1990) and Toräng *et al.* (2003), and it was observed that shifts in the biodegradation rates occurred when lower chemical concentrations were applied. Likewise, chemical concentration may affect the adaptation phase. Microbial communities require longer adaptation phase in the presence of low chemical concentrations, and adapted inocula with lower concentrations are not as efficient as pre-adapted inocula obtained with higher concentrations (Spain and van Veld, 1983).

Many chemicals have been identified as toxic to microbial inocula and may cause inhibition of the biodegradation processes. Radehaus and Schmidt (1992) showed that pentachlorophenol (PCP) at a concentration of 200 mg/L inhibited

cell growth of the PNP-degrader, *Pseudomonas* sp. strain R2 and biodegradation of PCP. Folsom *et al.* (1990) reported inhibition of phenol biodegradation by trichloroethylene. Al-Ahmad *et al.* (1999) demonstrated inhibition of *Pseudomonas putida* caused by antibiotics, with high toxicity recorded for sulfamethoxazole. Antibiotics and biocides have also been reported to affect the biological processes in Wastewater Treatment Plants (WWTPs) (Al-Ahmad *et al.*, 1999; Stasinakis *et al.*, 2008).

Effect of chemical concentration on biodegradation kinetics, microbial adaptation phase and chemical availability was discussed in Chapter 1 sections **1.6.5** and **1.6.6**. Chemicals may also induce changes in community structure of bacterial populations present in microbial inocula. Chemicals that serve as a single carbon source in biodegradation tests may affect the survival of specific degraders, when low chemical concentration is used (Harms and Bosma, 1997), and for this reason could impact on the overall community structure and its potential for biodegradation. Specific degraders may also be more abundant in the presence of higher chemical concentration due to the faster biodegradation of chemical and higher bacterial growth rate than in the presence of low or threshold chemical concentrations (Lewis *et al.*, 1986; Swindoll *et al.*, 1988). The concentration of test chemical is a key factor impacting the adaptation phase of microbial populations present in OECD inoculum, which may impact biodegradation kinetics and make the interpretation of results difficult to extrapolate to real world and use in risk assessment. This could also have consequences on the reproducibility of biodegradation studies. According to REACH guidance the results can be extrapolated to biodegradation in the real environment only if the concentrations that were used in the test are in the same order of magnitude as the concentrations that are to be expected in the real environment. Otherwise extrapolation can overestimate the biodegradation rates especially when the extrapolation goes from high to low concentrations leading to false positives (ECHA, 2012).

5.2. Objectives

The main objective of this work was to investigate the effect of *para*-nitrophenol (PNP) test concentration on the biodegradation of PNP and to find the threshold PNP concentration below which PNP biodegradation does not occur. The following hypotheses were tested: (1) PNP concentration affects the biodegradation of PNP, (2) a threshold concentration exists for PNP biodegradation and (3) PNP concentration affects the abundance of PNP-degrading bacteria, and consequently affects the potential for PNP biodegradation.

5.3. Materials and methods

5.3.1. Inoculum material

In May 2012 effluent biofilms were collected from River Dene (Chapter 3, **Figure 3.1** effluent discharge point (site C)). After eight days of glass slide colonization as described in Chapter 4 section **4.3.1**. Biofilm biomass was assessed using methods described in Chapter 4 section **4.3.3**.

5.3.2. Experimental set up

The experiment was conducted in sterile amber Duran Schott bottles (500 mL) which were filled with 300 mL of autoclaved river water. Treatments were prepared in triplicate and consisted of bottles with autoclaved river water inoculated with biofilm grown on three microscopic glass slides in WWTP effluent. Positive controls consisted of autoclaved river water inoculated with PNP-induced *Pseudomonas syringae* AKHD2 grown on LB medium with PNP (56 mg/L) for 48 hours at 25 °C. The negative controls consisted of sterile mineral medium. Sterilization was performed by autoclaving at 121 °C for 15 minutes at 1.1 atm. PNP was added to bottles to obtain four different concentrations: 2000, 200, 20, and 2 µg/L along with equal aliquots of ¹⁴C PNP stock solution. Incubation conditions were as described in Chapter 2. Initial incubation was for 28 days. After 28 days incubation was extended up to 54 days for replicates which failed to degrade PNP within the initial 28 day period. Samples for biodegradation measurements were taken every two days during the 28 day incubation period. After that, samples were taken weekly.

5.3.3. ^{14}C -labelled PNP and PNP-radiation and chemical concentration

Concentrated PNP solutions were prepared and 0.5 μL of each solution was transferred to appropriate bottles with 300 mL of sterile river water to obtain initial PNP concentration of either 2000, 200, 20 or 2 $\mu\text{g/L}$. ^{14}C -labelled PNP with specific activity of 55 mCi/mmol (American Radiolabeled Chemicals Inc., USA) stock was dissolved in 500 μL of sterile water and 15.6 μL of that stock was transferred to each bottle with 300 mL of sterile river water and the initial radioactivity was 0.6 kBq/mL. The amount of ^{14}C PNP added per bottle was 2.04 MBq/mol PNP which gave 2, 17, 67 and 95 % of total PNP present in treatments with 2040, 240, 60 and 42 $\mu\text{g/L}$ of PNP, respectively.

5.3.4. Monitoring the biodegradation of parent ^{14}C labelled PNP and PNP

5.3.4.1. Radio-HPLC

Daily 1 mL aliquots of medium were removed from each bottle and parent PNP concentration was measured by radio-HPLC. The analysis was carried out using a UV-1575 HPLC (Jasco, UK) with β -RAM radio-tracer with liquid flow cell (500 μL) (LabLogic, UK) and a LiChrosphere (5 μm) C-18 column (Merck Millipore, UK). The mobile phase was a mixture of water: methanol (HPLC grade, Fisher Scientific, UK) with a ratio of 40:60 and a flow rate of 0.50 mL/minute. The FlowLogic U scintillation flow liquid (LabLogic, UK) was supplied at a flow rate of 1.50 mL/minute. Analysis was performed with UV detection at 254 nm, with column pressure of 115 bar and column temperature at 25 °C. The volume of the injected sample was 100 μL . PNP standard solutions containing 2 – 2000 $\mu\text{g/L}$ of PNP were used for instrument calibration.

5.3.4.2. Scintillation counts

As described in Chapter 2 section **2.3.7.2**.

5.3.5. Microbial ecology methods

DNA was extracted from freshly collected biofilms (T_0) and at the end of incubation or after complete PNP biodegradation (T_{end}). DNA extraction was performed as described in Chapter 4 section **4.3.3.3**. TRFLP, and QPCR for PNP functional markers and the 16S rRNA gene were performed as described in Chapter 2 sections **2.3.9** and **2.3.15**, respectively.

5.3.6. Statistical analyses

As described in Chapter 2 section **2.3.18**.

5.4. Results

5.4.1. The effect of PNP concentration on the biodegradation rate of PNP

Successful PNP biodegradation was observed at a total PNP concentration of 2040, 240 and 60 $\mu\text{g/L}$ (**Figure 5.1**). ANOVA for the length of the lag phase, maximum PNP biodegradation rate and time needed for 50% PNP biodegradation (DT_{50}) (**Table 5.2**) showed no significant effect of PNP concentration at the concentration range of 60-2040 $\mu\text{g/L}$ on the biodegradation of PNP. Nevertheless, a threshold for PNP was seen in the range of 42 $\mu\text{g/L}$. Variable PNP biodegradation (0, 65 and 90% of applied PNP) was found among replicates ($n=3$) at the lowest PNP concentration. Application of such low concentration of PNP resulted in longer adaptation phase and longer incubation time was required to degrade 50% of applied PNP (**Table 5.2**). The maximum PNP biodegradation rate was not calculated due to the high discrepancy between replicates. It was also observed that positive control, PNP-induced isolate *Pseudomonas syringae* AKHD2, degraded PNP at all tested concentrations, with no detected lag phase prior to PNP biodegradation and with almost two times faster PNP biodegradation rate in comparison with river biofilms. Overall, slower PNP biodegradation rate was observed for *P. syringae* with decreasing PNP concentration (**Table 5.3**). Similar proportion of $^{14}\text{CO}_2$ were trapped (**Table 5.4**) during the biodegradation of different PNP concentrations with biofilms (**Figure 5.2**) but higher amount of $^{14}\text{CO}_2$ was trapped during the PNP biodegradation with *P. syringae* AKHD2 (**Table 5.4, Figure 5.2**).

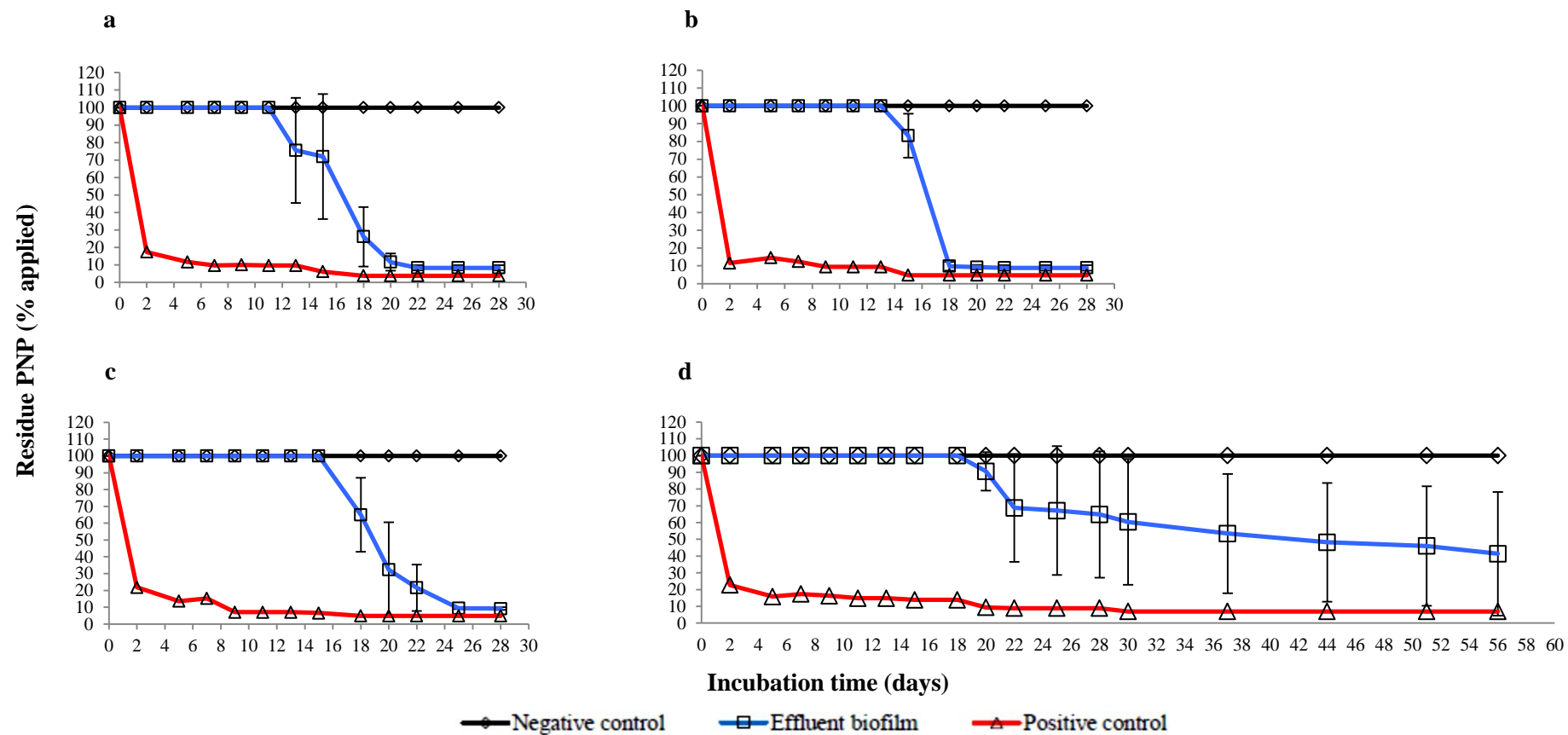


Figure 5.1: The biodegradation of ^{14}C PNP at different total PNP concentrations with effluent biofilm. Where ($\mu\text{g/L}$): a- 2040, b- 240, c- 60 and d- 42; error bars showing \pm standard error of the mean (S.E.M.), $n=3$

Table 5.2: Comparison of DT-values, length of lag phase and maximum PNP degradation rate for PNP biodegradation at different PNP concentrations with biofilms.

Total PNP concentration (µg/L)	Lag phase (days)	Max PNP degradation rate (% PNP/day)	Max PNP degradation rate (µg/L/day)	DT ₅₀ (days)
2040	13.03 (± 1.48) ^a	16.06 (± 2.85) ^a	321.00 (± 57.14)	16.17 (± 1.85) ^a
240	15.00 (± 0.50) ^a	15.33 (± 2.73) ^a	36.67 (± 1.33)	16.33 (± 0.17) ^a
60	17.33 (± 1.33) ^a	16.69 (± 4.63) ^a	3.33 (± 0.37)	19.33 (± 1.09) ^a
42*	34.67 (± 10.91) ^a	-	-	39.33 (± 10.14) ^b

Where: treatments with different letters are significantly different (P<0.05); * two replicates (n=3) degraded 90% and 65% of applied PNP. The incubation time (56 days) was used as a measure of lag phase and DT₅₀ for the replicate that did not degrade PNP (0%); ± standard error of the mean (S.E.M.); n=3.

Table 5.3: Comparison of DT-values, length of lag phase and maximum PNP degradation rate for PNP biodegradation at different PNP concentrations with *Pseudomonas syringae* AKHD2

Total PNP concentration (µg/L)	Lag phase (days)	Max PNP degradation rate (% PNP/day)	Max PNP degradation rate (µg/L/day)	DT ₅₀ (days)
2040	-	30.00	600.00	1.00
240	-	36.80	73.60	1.00
60	-	21.75	4.35	1.00
42	-	17.00	0.34	1.00

Where n=1.

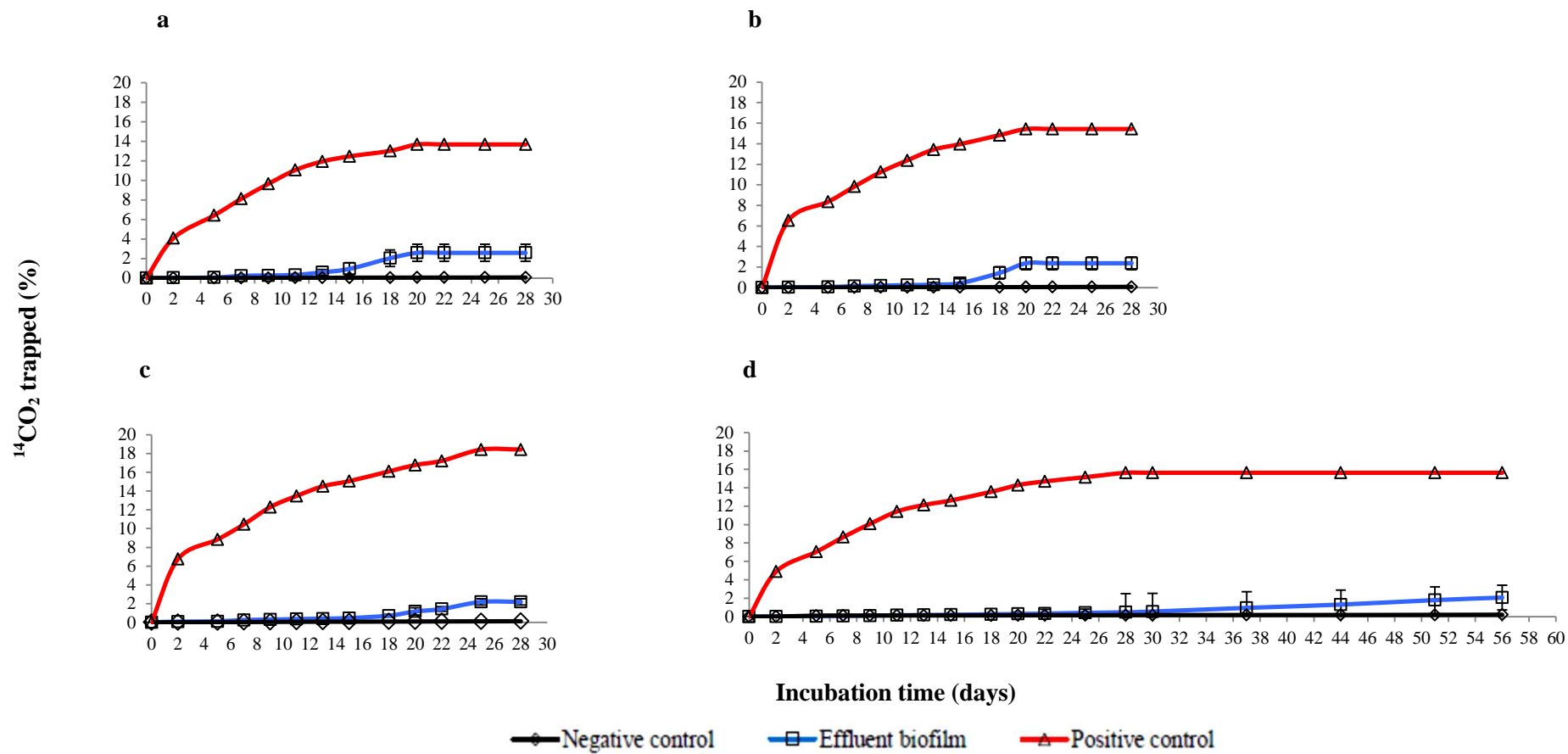


Figure 5.2: Release of $^{14}\text{CO}_2$ during the biodegradation of ^{14}C PNP at different total PNP concentrations. Where ($\mu\text{g/L}$): **a**- 2040, **b**- 240, **c**- 60 and **d**- 42; error bars are showing standard error of the mean (S.E.M.), $n=3$

Table 5.4: Trapped $^{14}\text{CO}_2$ during the biodegradation of ^{14}C PNP with biofilms and *Pseudomonas syringae* AKHD2.

Total PNP concentration ($\mu\text{g/L}$)	Biofilm	<i>Pseudomonas syringae</i> AKHD2
	$^{14}\text{CO}_2$ trapped (%)	
2040	2.58 (± 0.72)	13.68
240	2.37 (± 0.15)	15.43
60	2.18 (± 0.27)	18.43
42	2.07 (± 1.10)	15.63

Where: \pm standard error of the mean (S.E.M.), n=3

5.4.2. Biofilm biomass

Using assays described in Chapter 4 biomass of the biofilms was determined based on the EPS concentration i.e. glucose equivalents, number of culturable bacteria and concentration of extracted DNA. The biofilm biomass Table 5.5 was similar to the biomass levels presented in Chapter 4 for the May biofilms sampled one day before biofilms collection for the present experimental work.

Table 5.5. Inoculum size – biofilm biomass determined based on the DNA concentration, EPS concentration, and number of cultivable bacteria.

Date	Sampling site	DNA extraction (ng/L)	EPS as glucose equivalents ($\mu\text{g/L}$)	Bacterial counts (cfu/L)
May 2012	Effluent	89.24 (± 11.58)	266.32 (± 1.16)	6.69×10^6 ($\pm 3.33 \times 10^5$)

Where: \pm standard error of the mean (S.E.M.), n=3

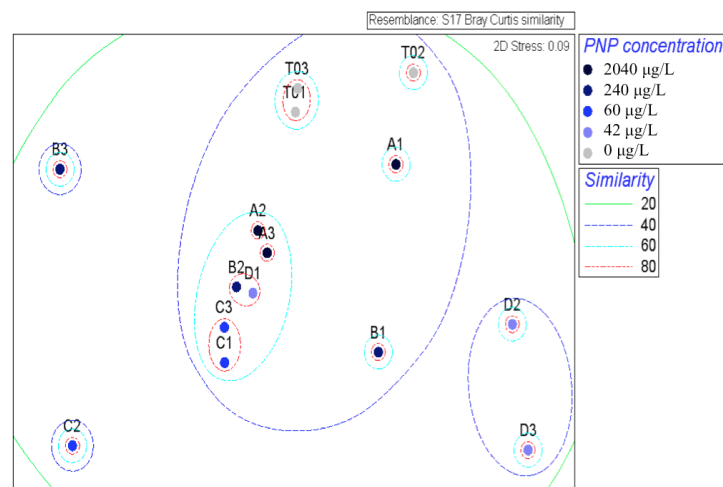
5.4.3. Effluent biofilm bacterial TRFLP profiles

5.4.3.1. All Terminal Restriction Fragments

High similarity (up to 60%) (**Figure 5.3 a**) for biofilm profiles incubated with different PNP concentrations was observed together with close clustering of

TRFLP profiles from different treatments (**Figure 5.3 b**). In addition, ANOSIM revealed no significant differences between the effluent biofilm TRFLP profiles after PNP biodegradation at different PNP concentrations. Also, no differences were found between T_0 biofilms and at the end of incubation. No statistical differences were found between treatments possibly due to high variation between replicates. It was noticeable that often one replicate was different to the others which may suggest the difficulty of sampling relatively rare organisms in relatively low concentration of biomass.

a



b

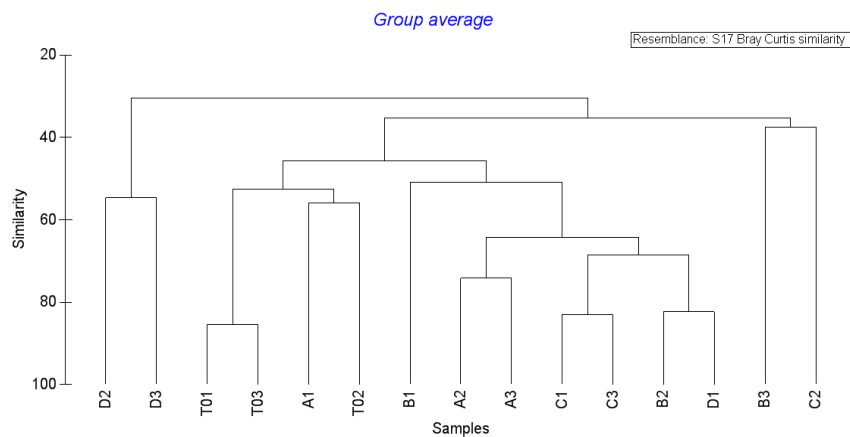


Figure 5.3: Analysis of TRFLP biofilm community profiles.

Figure legend: **a**- Non-Metric Multidimensional Scaling (NMDS) plot of TRFLP bacterial profiles, using Bray- Curtis distance for the data set derived from amplification with bacterial primers 63F and 1087R-VIC fluorescently labelled, and digested with *MspI* and *HhaI*. Stress: 0.09. **b**- dendrogram showing cluster analysis for TRFLP profiles. TRFLP profiles for fresh effluent biofilm (T_0) and biofilm incubated with PNP at a total concentration ($\mu\text{g/L}$): **A1-3**- 2040, **B1-3**- 240, **C1-3**- 60 and **D1-3**- 42.

Total number of Terminal Restriction Fragments (TRFs) present in effluent biofilm profiles was calculated and ANOVA showed no significant differences between the biofilm profiles (**Table 5.6**).

Table 5.6: ANOVA for total number of TRFs present in effluent biofilm TRFLP profiles before and after PNP biodegradation at different PNP concentrations.

Effluent biofilm and total PNP ($\mu\text{g/L}$)	Number of TRFs
0	48.33 (± 5.77)
2040	54.33 (± 7.06)
240	44.00 (± 4.04)
60	44.00 (± 7.09)
42	41.33 (± 9.61)

Where: \pm standard error of the mean (S.E.M.); n=3.

5.4.3.2. TRF 245 nt

The relative abundance of TRF 245 nt, (**Figure 5.4**) shown in Chapter 2 to represent the PNP-degrading *P. syringae*, was calculated for effluent biofilm profiles by determining the percentage of fluorescence intensity of this TRF relative to the sum of fluorescence intensity from all TRFs. ANOVA for the relative abundance of TRF 245 nt (**Table 5.7**) revealed no significant differences ($P>0.05$) for biofilms incubated with different PNP concentrations. No differences were found between the amount of TRF 245 nt present in fresh effluent biofilm and the amount detected after incubation with PNP.

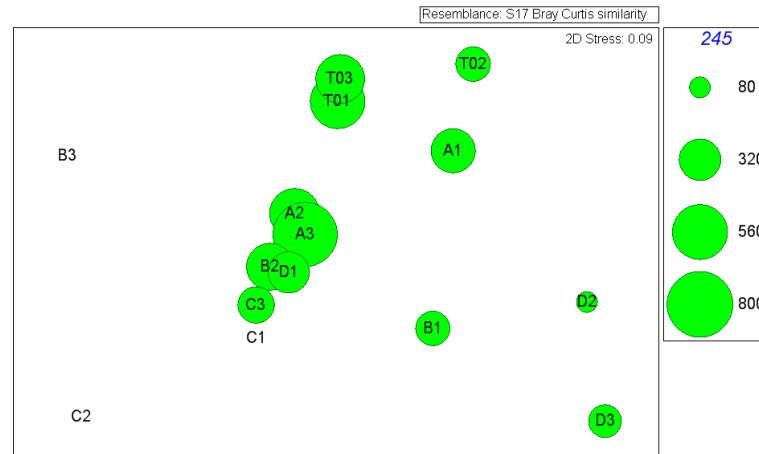


Figure 5.4: A 2D bubble plot presenting the amount of TRF 245 nt (TRF identified as *Pseudomonas syringae*) in environmental samples using Bray-Curtis similarity. Stress: 0.09. Figure legend: TRFLP profiles for fresh effluent biofilm (T_0) and biofilm incubated with PNP at a total concentration ($\mu\text{g/L}$): **A1-3-** 2040, **B1-3-** 240, **C1-3-** 60 and **D1-3-** 42. Green circles correspond with the amount of TRF 245 nt in TRFLP profiles.

Table 5.7: Relative abundance of TRF 245 nt in effluent biofilm TRFLP profiles before and after PNP biodegradation.

Effluent biofilm and total PNP ($\mu\text{g/L}$)	Relative abundance of TRF 245 nt
0	3.69 (± 0.53)
2040	1.85 (± 0.98)
240	0.64 (± 0.64)
60	2.93 (± 0.31)
42	1.78 (± 0.62)

Where: \pm standard error of the mean (S.E.M.); $n=3$.

5.4.4. QPCR for PNP functional genes and 16S rRNA gene

ANOVA for the gene copy number of *pnpA*, *mar* and 16S rRNA (**Figure 5.5 a, b and c**, respectively) showed significant differences for the copy number of *pnpA* and 16S rRNA gene. Higher *pnpA* and 16S rRNA gene copy numbers were detected in biofilms after PNP biodegradation at 2040 and 42 µg/L than in biofilms at T₀ and biofilms which degraded 240 and 60 µg/L PNP. It needs to be pointed out that copy numbers for *pnpA* also included the false positives and *mar* does not represent PNP-degraders only (as discussed in Chapter 2). Also, copy numbers for 16S rRNA gene are too low (possibly due to the long gene fragment), and hence, they might not represent the actual number of bacteria in the samples.

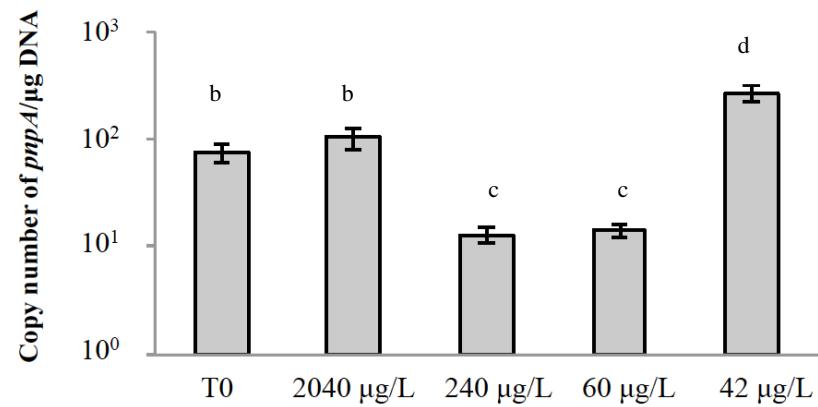
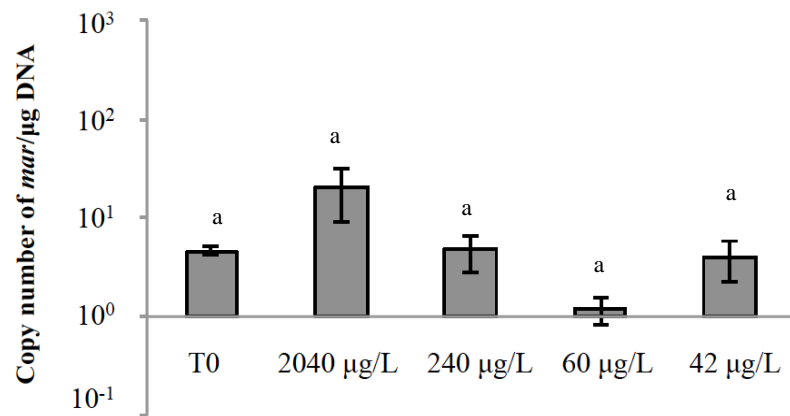
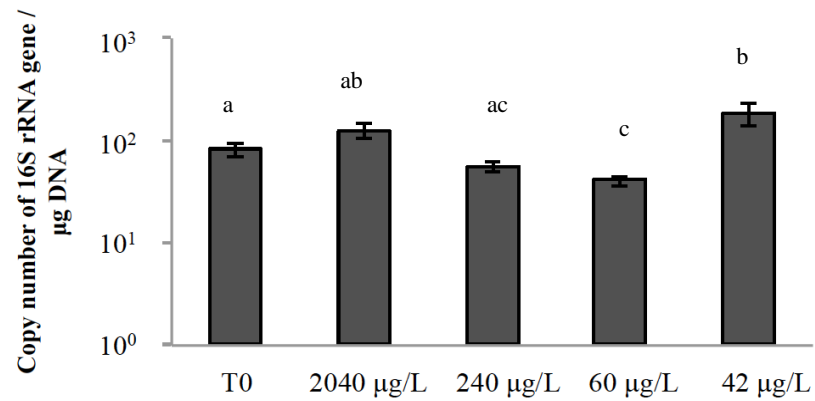
a**b****c**

Figure 5.5: Effect of PNP concentration on the copy number of PNP functional genes and 16S rRNA gene. Figure legend: a- *pnpA*, b- *mar*, c- 16S rRNA; treatments with different letters are significantly different ($P < 0.05$); error bars showing \pm standard error of the mean (S.E.M.), $n=3$.

5.5. Discussion

5.5.1. The effect of PNP concentration on the PNP biodegradation rate and threshold PNP concentration

Many organic chemicals are present at low concentrations in natural environments (Harms and Bosma, 1997). However, most of the biodegradation studies are conducted with high concentrations of test compounds (Ahtiainen *et al.*, 2003; Kolpin *et al.*, 2002). It is wrongly assumed that the reactions which occur at higher concentrations also take place at low concentrations. Hoover *et al.* (1986) reported anomalies which exist in mineralization of low concentration of organic compounds in lake water and sewage. Hoover *et al.* (1986) suggested that (1) the rate of mineralization is less predictable at lower chemical concentration; (2) chemicals mineralized at lower levels might not result in complete biodegradation; (3) biodegradation may not follow the commonly described kinetics; (4) compounds might be mineralized by some but not all natural inocula (Hoover *et al.*, 1986).

PNP is also present in natural environments at low concentrations with few µg/L of PNP measured in rivers (**Table 5.1**), which is in the range of described above threshold concentration. Hence, it is possible that PNP biodegradation may not occur at low PNP concentration in the natural environments. According to US Environmental Protection Agency (EPA) the PNP concentration in natural water should not exceed 10 ng/L. Many studies have been conducted to determine the effect of PNP concentration on biodegradation rates. For instance, it was found that with increased inoculum size acclimation period was shortened at low PNP concentration (26 µg/L) in two bacterial cultures of *Corynebacterium* sp. and *Pseudomonas putida* compared to that at a higher concentration of 1-10 mg/L. Also, the rate and extent of PNP mineralization were enhanced at lower PNP concentration (Zaidi *et al.*, 1996).

Based on the PNP biodegradation results (**Figure 5.1**, **Table 5.2**) obtained in the present study it could be suggested that higher concentrations of PNP seem to be inducing the biodegradation much faster than the lowest concentrations and there seems to be a threshold concentration below which the biodegradation of PNP will

not occur or the biodegradation rate will be lower after much longer adaptation phase. This is usually associated with the minimum level of organic chemical which is needed for induction of degradative genes and activation of biodegradation pathways (Hanne *et al.*, 1993; Zaidi *et al.*, 1996). Although, PNP-induced strain *Pseudomonas syringae* AKHD2 degraded PNP at a concentration of 42 µg/L, the biodegradation of PNP with biofilms demonstrated a threshold concentration below which PNP is not degraded and this was observed in the range of 42 µg/L. (**Figure 5.1**). It is also possible that a lesser extent of PNP biodegradation observed at concentration of 42 µg/L could be due to the nutrient depletion over the incubation time which was extended to 56 days. Low level of nutrients affects the functionality of microbial populations, and therefore, could lead to the limited chemical biodegradation (Joner and Leyval, 2003) while shorter lag phase, and chemical biodegradation rate were observed in biodegradation systems amended with nutrients (Gotvajn and Zagorc-Končan, 1999). It is noteworthy that the amount of $^{14}\text{CO}_2$ trapped was higher during the biodegradation of PNP with *P. syringae* compared to biofilms, which could be due to the faster growth of PNP-induced *P. syringae* in the presence of parent ^{14}C PNP or higher fraction of ^{14}C PNP was assimilated into biofilm biomass.

Interestingly, a threshold concentration as low as 10 pg/L PNP was found for the adaptation prior to PNP biodegradation in freshwater samples by Spain and van Veld (1983) while Schmidt *et al.* (1987) reported 10 µg/L as a threshold for PNP biodegradation with *Pseudomonas* sp. The lack of PNP biodegradation at low PNP concentration (0.2 µg/L) was also demonstrated in Chapter 2 using PNP-degrading bacterial strains: *Arthrobacter* sp. JS443 and *Pseudomonas syringae* isolate AKHD2. Although, no biodegradation of PNP was observed at 20 µg/L with river water, difficulties were encountered in measuring the PNP concentration at this level using HPLC method described in Chapter 2 section 2.3.3. In the current Chapter application of the ^{14}C PNP assay together with radio-HPLC enabled detection of PNP biodegradation at a concentration of 2 µg/L. It was demonstrated that effluent biofilms degraded PNP at a concentration of 20 µg/L within a 28 day incubation window, which is a requirement for OECD ready biodegradation tests. However, inconsistent and a lesser extent of PNP biodegradation at 2 µg/L within 28 days of incubation could suggest that application of higher tier OECD tests (e.g. inherent

biodegradability) would be more suitable to determine the biodegradation of PNP by natural inocula at such low PNP concentration.

5.5.2. Effect of PNP concentration on effluent biofilm TRFLP profiles, abundance of PNP-degraders and potential for PNP biodegradation

In the present study no effect of PNP concentration was found on the biofilm bacterial community structure (**Figure 5.3**) and potential for PNP biodegradation was not affected by a total PNP concentration in the range of 60-2040 µg/L (**Figure 5.1**). Although, biofilm communities had difficulties with the biodegradation of lowest PNP concentration (42 µg/L) within 28 days of incubation (**Figure 5.1**), similar bacterial TRFLP profiles were present in biofilms degrading different PNP concentration. This might suggest that PNP was not toxic to biofilm inoculum at studied concentrations, and therefore, no significant changes were observed in the bacterial community structure (**Figure 5.3**) and abundance of TRF 245 nt (**Figure 5.4** and **Table 5.7**) previously identified as PNP-degrading *Pseudomonas syringae*. There was also no correlation between the number of copies of both PNP functional markers at the end of the incubation and the concentration of PNP applied (**Figure 5.5**). However, non-target hits present in QPCR for *pnpA* and *mar* and low 16S rRNA gene copy numbers may suggest that these counts do not represent the actual abundance of bacteria in the samples. Overall, in the current project it was initially expected that the abundance of PNP degraders together with the number of copies of PNP functional genes would increase with increasing PNP concentration, since higher PNP concentration would lead to growth-linked PNP biodegradation, whereas longer incubation time and lower biomass production would occur at low PNP concentration.

Given the high variability of replicates within treatments, it is difficult to state whether PNP concentration had a significant impact on bacterial community composition present in biofilm inocula. Hence, it was not possible to determine if presented results support or are opposed to initial hypothesis. Although, the effect of higher chemical concentrations on bacterial growth and chemical biodegradation has

been well studied, there is limited information on the impact of lower concentrations on biodegradation results, except for the existence of threshold concentrations for chemical biodegradation. Some authors studied the diversity of bacteria naturally occurring at contamination sites. Loss of bacterial diversity as a result of biodegradation of high benzene concentration in ground water was reported by Fahy *et al.* (2005) and the abundance of certain groups of chemical degrading-bacteria was found to be much higher in the highly polluted sites by Hernandez-Raquet *et al.* (2006). However, no effect of contamination level on the biodiversity of microbial communities was observed (Hernandez-Raquet *et al.*, 2006). There are also reports on the inhibitory effect of high chemical concentrations on the inocula used in biodegradation tests. Triclosan (TCS) is a known inhibitor of nitrifying communities present in activated sludge, and at a concentration of mg/L it impacts on microbial community function i.e. ammonia removal is impacted. As a consequence the efficiency of biodegradation processes occurring e.g. in wastewater treatment may be affected (Stasinakis *et al.*, 2008). Comber and Holt (2010) also indicated that high chemical concentrations may be toxic to microbial inocula, and this could be the reason of false negative results in biodegradation tests.

5.6. Conclusions

The following conclusions can be drawn from the present study:

1. The biodegradation of PNP is affected by concentration and it could be suggested that a threshold PNP concentration for PNP biodegradation is around 42 µg/L.
2. There is an evidence that PNP biodegradation is possible at 42 µg/L.
3. High variability of replicates within treatments caused difficulties with data interpretation, and therefore, it was impossible to determine if PNP concentration affected microbial community composition.
4. There was no correlation between PNP concentration and the number of copies of both functional markers *pnpA* and *mar* present after complete PNP biodegradation.
5. ¹⁴C PNP assay was required for monitoring the biodegradation of PNP at concentrations lower than 42 µg/L.
6. Since PNP biodegradation at a concentration of 42 µg/L required longer than 28 day incubation, higher tier OECD tests could be suggested for studying the biodegradation of PNP at low concentration.

Chapter 6

General discussion

The present PhD project aimed to improve our understanding of biodegradation mechanisms occurring in rivers, and to identify bacterial populations involved in these processes in order to identify factors which could increase the realism and reproducibility of biodegradation tests. An attempt was made to introduce novel approaches (**Figure 6.1**) into biodegradation test systems and standardized procedures for inoculum collection and characterization prior to biodegradation testing to improve chemical risk assessment. The major findings of the current project are discussed below.

6.1. Variability of microbial inocula

Microbial inocula may vary in terms of type of material and location from which they are collected, which may impact biomass and community composition, and hence, functional capabilities. OECD tests lack a definition of inoculum source and quality which is recognized as a major source of inconsistent test results (Vázquez-Rodríguez *et al.*, 2006). Major benefits of standardizing inoculum are comparability of tests and reduced failure rate for these tests.

In order to characterize inoculum prior to biodegradation tests several methods were developed in the current project. Chapter 4 demonstrated preparation of biofilm slides, methods to quantify inocula and their catabolic potential. The reproducibility of these methods across sampling site and sampling date in experimental work conducted from November 2011 until May 2012 was also presented. In Chapter 3 river water, sediment extracts and Wastewater Treatment Plant (WWTP) effluent were also used as inocula. Experiments were focused on the biodegradation of *para*-nitrophenol (PNP) with these different inocula. Due to high variability of replicates it was difficult to determine whether inoculum material and sampling time had an impact on the biodegradation rate of PNP. However, it was revealed that

Wellesbourne WWTP effluent discharge point is a sampling site which provides consistent inoculum for biodegradation tests with sufficient bacterial biomass. Biofilms as well as river water collected from this site consistently degraded PNP. In addition, biofilm inoculum enabled concentration of bacterial biomass (increased inoculum size).

Inoculum density has been reported by Thouand *et al.* (1995) to be an important issue in chemical biodegradation tests, since inoculum size affects the outcome of biodegradation tests. The OECD guidelines for ready biodegradability indicate that the cell density in the test medium should initially be no more than 10^6 cells/mL (Forney *et al.*, 2001). In the current project the size of biofilm inoculum was within the range of 10^6 - 10^7 cfu/mL cultivable bacteria, while the number of bacteria in river water was from 10^3 to 10^6 cfu/mL, and varied between sampling times (**Table 6.1**). Although, no correlation was found between the inoculum size and the rate of PNP biodegradation in the biodegradation experiments described in Chapter 3 and 4, it should be pointed out that there are cases in which PNP biodegradation tests conducted with river water inoculum (experiments indicated in **Table 6.1**) and lake water reported by Ramadan *et al.* (1990) have failed to degrade PNP or PNP biodegradation occurred after a much longer adaptation phase when inocula with low numbers of bacteria were used (**Appendix Figure 0.16-0.18**).

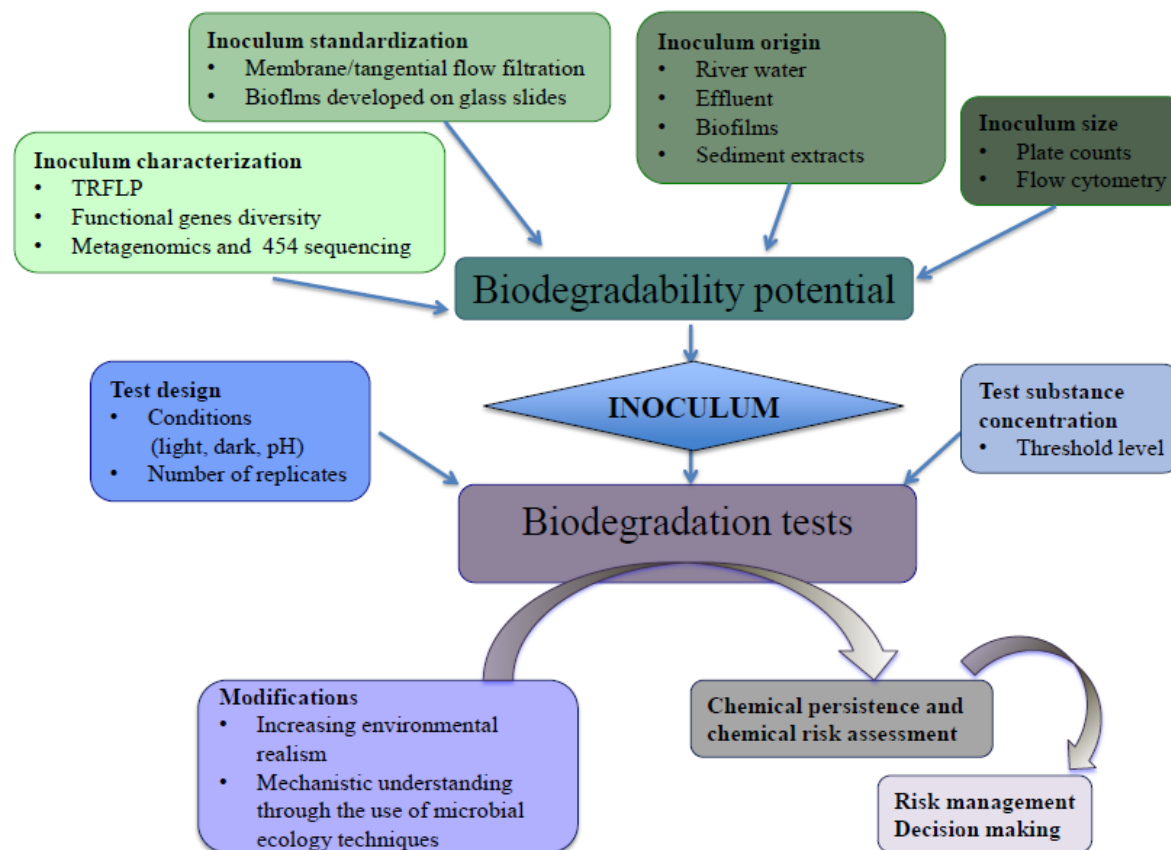


Figure 6.1: New approaches for the biodegradation potential assessment prior to biodegradation testing and improved risk assessment for chemicals.

Table 6.1: Inoculum size in different biodegradation experiments.
(Inocula which failed to degrade PNP are indicated with *)

Sampling time	Inoculum source	Inoculum size (cfu/mL)	Aim of experiment	Experiment No.	DT ₉₀ (days)
March 2010	river water downstream	3.68x10 ⁴	Effect of light/dark	1	No degradation/8
	river water upstream	1.53x10 ⁶			
	effluent	1.03x10 ⁵			
November 2011	river water downstream	2.57x10 ⁶	Effect of inoculum source	2	8
	sediment extract upstream	8.59x10 ⁶			
	sediment extract downstream	5.23x10 ⁶			
June 2011*	river water downstream	8.84x10 ³	Effect of light	3	No degradation
July 2011*	river water downstream	6.40x10 ³	¹⁴ CPNP-threshold concentration	4	No degradation
	biofilm upstream*	1.22x10 ⁷			No degradation
November 2011	biofilm effluent	4.22x10 ⁷	Variation of biofilm inocula	5	28
	biofilm downstream*	2.38x10 ⁷			No degradation
January 2012*	river water downstream	6.19x10 ⁴	¹⁴ CPNP- threshold concentration	6	84
	biofilm upstream	1.68x10 ⁶			
February 2013	biofilm effluent	1.18x10 ⁷	Variation of biofilm inocula	7	12
	biofilm downstream	3.43x10 ⁵			
	river water+effluent	9.04x10 ⁴			
	biofilm upstream	1.28x10 ⁶			
May 2012	biofilm effluent	1.68x10 ⁶	Variation of biofilm inocula	8	13
	biofilm downstream	2.08x10 ⁷			
	river water + effluent	3.00x10 ⁶			
May 2012	biofilm effluent	6.69x10 ⁶	¹⁴ CPNP-threshold concentration	9	56

6.2. Inoculum characterization

Inoculum characterization is also beneficial since it may enable us to identify the reason for test failure and provide a mechanistic understanding of biodegradation processes. It is also useful for the comparison of test results obtained with inocula sampled from different environmental compartments. Forney *et al.* (2001) indicated that inoculum density may be used to predict chemical biodegradation, since high inoculum density is related with higher abundance of specific degraders. In the present project the following culture dependent and independent approaches were used; quantification of inoculum size, inoculum diversity and biodegradation potential based on Terminal Restriction Fragment Length Polymorphism (TRFLP) and functional assays for *pnpA* and *mar*.

Experimental work in this section aimed to determine bacterial community composition within inocula and changes which appeared in these communities after PNP biodegradation. Chapter 2 demonstrated the effect of light and dark conditions and the biodegradation of PNP on river water bacterial community structure. In Chapter 3 a range of PNP biodegradation tests were also conducted with different inocula such as sediment extracts, river water, and effluent, while in Chapter 4 experimental work was focused on the spatial and temporal variability of biofilm inocula. Inoculum characterization was applied prior to biodegradation tests and the results indicated that bacterial community composition is affected by such environmental factors as light/dark conditions, inoculum material and sampling time. Number of copies of PNP functional markers *pnpA* and *mar* were also affected by these factors but it is noteworthy that the number of copies of functional markers does not necessary reflect the number of true sequences of functional genes (Chapter 2). False positives were present in copy number for *pnpA*, while *mar* functional marker targeted other than PNP-degrading bacteria. Hence, the overall result (copy number) indicated much higher biodegradation potential than actually occurred. QPCR for copy number of degradative genes is not therefore the best method to study the biodegradation potential of communities present in environmental samples. Application of in depth analysis of functional markers (high throughput sequencing) would be a more accurate approach to study in a more detailed way the diversity of

functional genes, and to assess the presence of chemical degrading populations in test inocula.

Inoculum characterization performed in the current project enabled comparison of river water and biofilm bacterial community structure. Collected river biofilms were found to be representative of microbial populations abundant in flowing river water. It was also demonstrated that the majority of PNP-degrading bacteria detected in the current project belonged to the genus *Pseudomonas* which is widespread in the environment and has potential for biodegradation of a variety of chemicals (Spanggord *et al.*, 1991; Reardon *et al.*, 2000) including PNP. TRFLP data, and sequencing for PNP functional markers, *pnpA* and *mar*, indicated that *Pseudomonas syringae* in the key PNP-degrader and it was detected in river water, sediment, effluent and river biofilms. However, the biodegradation of other chemicals e.g. polycyclic aromatic hydrocarbons (PAHs) or polychlorinated biphenyls (PCBs) can require complex microbial communities and microbial synergism is essential for the combined metabolic attack at different sites on the organic compound, leading to eventual biodegradation of such complex chemicals (Borja *et al.*, 2005; Li *et al.*, 2008). Several authors have pointed out that community structure determines its function (Carlson *et al.*, 2004; Johnson *et al.*, 2004; Kirchman *et al.*, 2004) since presence of specific bacteria may be essential for chemical biodegradation. In some cases the persistence of specific degraders in the community may require the presence of other bacterial species providing e.g. nutrients, additional substrates, etc. (Sørensen *et al.*, 2002). Hence, microbial community structure in terms of the abundance of specific degraders may affect test results. Analysis of the local and metacommunities of biofilms, which was demonstrated in Chapter 4, revealed changes in the persistence and distribution of the key PNP-degrader *Pseudomonas syringae* in river microbial populations and these results correlated with PNP biodegradation test results. Overall biofilm metacommunities which degraded PNP had a high persistence of *P. syringae* which was identified as a core taxon after PNP biodegradation. Lack of PNP biodegradation corresponded with decreased persistence of *P. syringae* and changes in its distribution from a core to a satellite taxon. ‘Biodegradation lottery’ has been suggested as the main reason for test failures due to uneven distribution of specific degraders within the aliquots of inoculum used in biodegradation test. Although the ‘biodegradation lottery’ was

observed for some biofilms in Chapter 4, analysis for core and satellite taxa indicated that the proliferation of specific degraders is crucial for successful PNP biodegradation. Therefore, there is a possibility of microbial interactions within inoculum which could be responsible for proliferation of specific degraders in microbial populations and which might impact on biodegradation test outcome.

6.3. Test design

It is crucial to design biodegradation tests in a way which would enable the investigation of the fate of chemicals in the environmental compartment of interest. The key thing that should be considered in biodegradation test design is the purpose of biodegradation studies and whether the successful biodegradation of chemical is more important than the realism of the laboratory based tests. According to OECD test guidelines the purpose of biodegradation studies is to determine whether the chemical will undergo biodegradation in the environment. To assess this, the extent and the rate of chemical biodegradation are determined in laboratory tests and these results are extrapolated into natural environments.

The biodegradation studies should ideally be environmentally realistic, which means that they should consider natural conditions e.g. light/dark cycle, light intensity, bacterial community structure and functions present in applied inocula, their variability and consequences regarding the biodegradation test results. Only results obtained in more environmentally realistic studies could be used for better prediction of chemical fate in the environment, chemical risk assessment and management by the Chemical Industry. The best way of improving realism is to integrate the relevance of high dose experiments in terms of chemical biodegradation and microbial biodegradation mechanisms. In the present project experiments were conducted under more realistic conditions than current OECD tests, which involved the biodegradation of PNP under light and dark conditions in Chapter 2, and the biodegradation of PNP at environmentally relevant concentrations in Chapter 5. Increased river water pH under light conditions inhibited PNP biodegradation and a threshold PNP concentration was found for biodegradation. Although application of light in biodegradation test systems would increase their environmental realism, light

conditions lead to variable test results. Due to the growth of algae in small scale closed experimental systems chemical uptake and biodegradation by bacterial populations can be easily disturbed. Such a scenario would not take place in open systems like rivers unless they were heavily eutrophic. Lower levels of chemical compound might also be an issue in the biodegradation systems. Despite the fact that the studies conducted at lower chemical concentrations give more realistic results, detection of the test compound at very low levels could be challenging due to methodological limitations i.e. limit for chemical detection and background interference. In the current project complete PNP biodegradation was observed at a PNP concentration range of 60-2040 $\mu\text{g/L}$ with biofilm inoculum. The PNP concentration present in the natural environment was previously reported in the range of ng- $\mu\text{g/L}$ (BUA, 1992; Tan and Chong, 1993) which is below the threshold PNP concentration 42 $\mu\text{g/L}$ (Chapter 5). Hence, it would be useful to further investigate the fate of this chemical compound in river water, sediment and soil and to determine the realistic threshold concentration for PNP biodegradation.

6.4. Methods to characterize biodegradation potential

Biodegradation potential is an essential characteristic of inoculum. It helps to predict biodegradation rate of diverse chemicals, to determine degree of pre-exposure to chemicals within inoculum, it enables the cross comparability of results between chemicals, environmental compartments and experimental samples, and is crucial for understanding test failures. For instance, Federle *et al.* (1990) demonstrated that number of bacteria and bacterial community structure impact on an inoculum's biodegradation potential. A range of methods can be applied to determine the biodegradation potential of inocula. In the current project analysis of the functional markers *pnpA* and *mar*, including targeted next generation sequencing, was applied. These tools can be used to analyse DNA samples collected from a variety of environmental compartments (river water, sediment, WWTP's effluent, river biofilms) and to determine the impact of environmental factors on the biodegradation of PNP with river water and biofilm inocula. Functional marker analysis presented in Chapter 2 demonstrated how powerful these techniques are enabling detection and identification of bacterial populations involved in the biodegradation of PNP.

Regardless of application of high throughput sequencing and QPCR methods it was hard to determine the biodegradation potential of applied inocula and to predict how different factors e.g. inoculum material, sampling date, microbial biomass may impact on the biodegradation processes occurring in rivers i.e. the biodegradation of PNP. However, it was found that river water inocula which had lower than 10^5 bacterial cell counts were more prone to fail to biodegrade PNP. River biofilms appeared as a good inoculum source with high biomass density which resulted in consistent biodegradation of PNP with those inocula. Data collected during this project indicated that inoculum biodegradation potential depends on inoculum material and it varies between sampling dates. Extrapolation of laboratory test results into the environment i.e. rivers is still challenging mainly due to their complex nature and the many parallel processes occurring in the environment. Further experimental work should be focused on both seasonal and biogeographical differences and how they could affect the biodegradation potential of tested inocula.

6.5. Future recommendations

An overview of factors affecting biodegradation potential of tested inoculum and its effect on the biodegradation results is presented in **Figure 6.1**. The current project aimed to increase the realism of biodegradation tests. Based on PNP biodegradation experiments the following modifications could be suggested to standardized biodegradation tests:

a) increasing number of replicates

OECD test guidelines indicate that biodegradation studies should consist of two replicates of each treatment, and in practice experimental designs do not consider higher numbers of replicates. In the current project several PNP biodegradation experiments resulted in PNP biodegradation in one or two out of three replicates of the same treatment which could be related to ‘biodegradation lottery’. Therefore, increasing the number of replicates to four per treatment would give the highest chance of reproducible biodegradation results within the treatment and the possibility of statistical analysis of collected data (e.g. only treatments in triplicates can be used for NMDS analysis of TRFLP bacterial profiles).

b). variability of replicates

High variability of replicates lead to difficulties with statistical analysis in Chapter 3 and 5, and it was hard to determine whether there are differences between treatments. It appeared that there were often few statistical differences between samples because they varied greatly not because they were very similar. ANOVA was not a suitable method for statistical analysis of highly variable replicates. More appropriate analysis would require use of transformations and non-parametric tests.

Variation between replicates of TRFLP profiles also indicates difficulties with sampling representative microbial communities of given environment. Some species can be in low abundance, especially in low biomass samples and therefore, they might be difficult to detect using microbial ecology methods.

c) negative and positive controls

Controls should be included in the biodegradation tests along with the treatments to make sure that the test system operates correctly and to exclude any potential contamination which may affect the biodegradation results, and lead to incorrect data interpretation. Controls should also be used to determine the impact of different factors on the biodegradation processes e.g. effect of incubation conditions on bacterial community composition in river water inoculum.

d) realism of tests

Light conditions, light/dark cycle and test substance concentration (threshold concentration) should be considered in the experimental design. In the current project increased realism of tests lead to variable test results. Although, unrealistic conditions could give a false indication of chemical persistence in the natural environment, it is still recommended to conduct screening biodegradation tests in the dark/diffuse light conditions to avoid possible interference from algal growth and photosynthesis occurring in the light conditions.

e) inoculum characterization

Application of culture dependent methods to determine the size of inocula used in biodegradation studies (e.g. plating) and for isolation of PNP-degraders is a simple

standard procedure used for the assessment of biomass content and the presence of PNP-degrading bacteria in a variety of inocula. Application of culture independent methods to determine microbial community composition within inoculum (TRFLP) and their functional diversity (functional gene assay, high throughput sequencing) enabled in depth analysis of catabolic bacterial populations. Moreover, inoculum material and time when it was collected should also be taken into account.

6.6. Future work

6.6.1. Inoculum variability

Inoculum characterization, together with justified choice of test inoculum (biomass content, material, biodegradation potential) would help to achieve more reproducible biodegradation results. In the present project variation of environmental conditions was observed at different times of the year and biofilm community structure was significantly affected by these changes. Consequently inoculum potential for chemical biodegradation was also altered. For the global relevance of these results experimental work on chemical biodegradation using river inocula (sediment, water and biofilm) could be expanded to other rivers in the UK, and compared between different geographical locations e.g. rivers in UK and China. The experimental work should address:

- biodegradation tests with different inocula from different rivers
- variation of inocula by season and location
- comparison of spatial and temporal results

6.6.2. Functional marker approach

Microbial ecology techniques were found useful for inoculum characterisation. TRFLP including the abundance of *P. syringae* (TRF 245 nt), and functional markers analyses enabled detection of PNP degrading bacterial populations and identification of additional uncultivable degraders, respectively. Therefore, these

methods should be applied more routinely as standardised procedures prior to biodegradation tests and also to study chemical-degrading bacteria at the end of biodegradation/incubation. Studying shifts in bacterial community structure, presence and absence of specific species could be beneficial for understanding test failures. To expand our knowledge of the functional diversity of chemical-degrading bacteria further experimental work on the *mar* functional marker would be useful. Involvement of *mar* in the biodegradation of different chemicals was already reported by Seibert *et al.*, (1993). But the presence of *mar* in the biodegradation of chemicals which are of Industrial interest e.g. triclosan requires further experimental work. Application of such a marker would be beneficial for future biodegradation studies which involve a variety of chemicals. Future biodegradation studies could investigate:

- Biodegradation of a variety of chemicals i.e. readily biodegradable, with inherent biodegradability and resistant to biodegradation
- Application of microbial ecology methods e.g. high throughput sequencing for the detection of *mar* functional marker and analysis of *mar* sequences to determine the diversity of chemical-degrading bacterial populations

6.6.3. The use of the biodegradation studies and decision making

6.6.3.1. Persistence of chemicals in the environment

Microbial adaptation and biodegradation processes play a key role in chemical fate in the environment. Persistence of chemicals in the natural environment is usually assessed through biodegradation studies with microbial inocula. The chemical biodegradation test results are used to derive the rates of chemical biodegradation. The biodegradation tests also provide information on the chemical persistence which is essential for chemical risk assessment. Based on the risk assessment risk management and decision making is performed.

6.6.3.2. Modelling

Obtained datasets from biodegradation studies could be applied into models simulating different environmental scenarios which would improve our understanding of processes occurring in the environment and to predict potential chemical persistence. Increased knowledge on behaviour of chemicals in the environment would be beneficial for chemical risk assessment conducted by the Chemical Industry.

A mechanistic model is currently being developed at Unilever SEAC to simulate the OECD 314E test system. The model couples water quality parameters (COD, NH_3) and microbial growth (heterotrophic and autotrophic biomass) with the fate of chemicals. When key test variables (COD, NH_3) influencing test chemical biodegradation are described and parameterized within the model, fitted degradation kinetics parameters can be estimated for use in in-river exposure modelling. Further experimental work is required to enable the model to better characterize the microbial populations involved in the removal of wastewater constituents and, possibly in the removal of the test chemical.

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Appendices

List of appendix tables

Appendix Table 0.1: Primers designed for PNP functional gene <i>pnpA</i>	269
Appendix Table 0.2: Primers designed for PNP functional gene <i>npdA2</i>	270
Appendix Table 0.3: Primers designed for PNP functional gene <i>mar</i>	271
Appendix Table 0.4: Number of 454 reads for <i>pnpA</i> and <i>mar</i> gene.	272
Appendix Table 0.5. Variation in bacterial communities based on Index of Multivariate Dispersion (IMD).	273
Appendix Table 0.6: Slopes of rank-abundance plots for biofilm metacommunities. .	274

List of appendix figures

Appendix Figure 0.1: Diagram of OECD 314 with the most common transport pathways for chemicals discharged to wastewater.	275
Appendix Figure 0.2: Neighbour-joining tree showing <i>pnpA</i> gene database used for primer desing.....	276
Appendix Figure 0.3: Neighbour-joining tree showing <i>npdA2</i> gene database used for primer desing.....	277
Appendix Figure 0.4: Neighbour-joining tree showing <i>mar</i> gene database used for primer desing.	278
Appendix Figure 0.5: Example of bacterial 16S rRNA gene TRFLP profiles.....	279
Appendix Figure 0.6: Rank-abundance plots for local biofilm communities in November 2011 at T_0	280
Appendix Figure 0.7: Rank-abundance plots for local biofilm communities in November 2011 at T_{end}	281
Appendix Figure 0.8: Rank-abundance plots for local biofilm communities in February 2012 at T_0	282
Appendix Figure 0.9: Rank-abundance plots for local biofilm communities in February 2012 at T_{end}	283
Appendix Figure 0.10: Rank-abundance plots for local biofilm communities in May 2012 at T_0	284
Appendix Figure 0.11: Rank-abundance plots for local biofilm communities in May 2012 at T_{end}	285
Appendix Figure 0.12: Rank-abundance plots of meatcommunities.	286
Appendix Figure 0.13: Distribution and dispersal of bacterial taxa across TRFLP profiles from November biofilms.....	287
Appendix Figure 0.14: Distribution and dispersal of bacterial taxa across TRFLP profiles from February biofilms.	288

Appendix Figure 0.15: Distribution and dispersal of bacterial taxa across TRFLP profiles from May biofilms.....	289
Appendix Figure 0.16: The biodegradation of PNP at concentration of 2mg/L in the dark incubated river water and light incubated river water with variable pH (July 2011).....	290
Appendix Figure 0.17: ¹⁴C PNP biodegradation at PNP concentration of 2mg/L with river water inoculum (July 2011).	291
Appendix Figure 0.18: ¹⁴C PNP biodegradation at PNP concentration of 2mg/L with river water inoculum (January 2012).....	292

Appendix Table 0.1: Primers designed for PNP functional gene *pnpA*.

Name ^a	Sequence (5' → 3') ^b	Length (bp)	GC (%)	T _m (°C)
pnp193F	GARYCAGGCGTYTCRCCTTC	20	50-70	52-60
pnp223F	GTCGCCTACATGCCACCSAC	20	65	58
pnp277F	GACATYCGCAARCGYGCSGT	20	55-70	54-60
pnp277R	ACSGCRCGYTTGCGRARGTC	20	55-75	54-62
pnp1566R	SACACGKCGRCCATTCAAYTTT	22	41-55	51-57
pnp1570R	WATSACACGKCGRCCATTCA	20	45-55	50-54
pnp111AF	GTBGTDGTHGGCGSNGGYCC	20	60-85	56-66
pnp114AF	ATNGTHGGYGCNGTHCCNWC	20	45-75	50-62
pnp1083AR	RTGBGCVGCRTC BGCCAT	18	50-78	48-59
pnp1122AR	CAAMCCMAGDCCRCCVAWVG	20	45-75	50-62
pnp111AF	GTBGTDGTHGGCGSNGGYCC	20	60-85	56-66
pnp114AF	ATNGTHGGYGCNGTHCCNWC	20	45-75	50-62
pnp224F	TCGCCTACATGCCACCSACCG	21	67	60
pnp349F	AACGGYGARCTGATCGCGAAGAT	23	48-57	55-59
pnp535F	GTCGAGGAARTYGACCAGGAC	21	52-62	54-58
pnp635R	GTGCTGCGRGCRCCGTCGGT	20	70-80	60-64

^a – numbers at the end of the primer name refer to their relative position on the *pnpA* gene from strain *Pseudomonas* sp. WBC-3; ^b - sequences are of the sense strand

Appendix Table 0.2: Primers designed for PNP functional gene *npdA2*.

Name ^a	Sequence (5' → 3') ^b	Length (bp)	GC (%)	T _m (°C)
npd94F	TAYYTVGARWSBYTHCGMGA	20	25-65	42-58
npd190F	GCNATNGCNGARTAYTAYGA	20	30-60	44-56
npd274F	TGGTTCYTACCYBSGMTCAA	21	38-62	49-58
npd277F	CAYTGGTTCYTRCCNCGNTC	20	45-70	50-60
npd430R	GGNGTNCCRTTAAAYTTYTC	20	30-60	44-56
npd435R	CTTYTCRAACATYTCRTG	18	28-50	39-48
npd1362R	GTCRACCTGRTAYTCRGTC	20	45-65	50-58
npd1369R	CTGRTAYTCRGTCGCTGCATSGT	23	50-63	57-63
npd1441R	TTCRCTGATYTGMCGGAADAT	21	33-52	47-54

^a – numbers at the end of the primer name refer to their relative position on the *npdA2* gene from strain *Arthrobacter* sp. JS443; ^b - sequences are of the sense strand.

Appendix Table 0.3: Primers designed for PNP functional gene *mar*.

Name	Sequence (5'→ 3')	Length (bp)	GC (%)	T_m (°C)
mar349AF	CCNACSACSTAYGCVGGNAG	20	55-75	54-72
mar349BF	CCNACSACSTAYGCVGGNTC	20	55-75	54-72
mar715AR	RTGRCAVAGYTTGTGRTG	18	33-61	41-53
mar715BR	RTGRCAGATYTTGTGRTG	18	33-56	41-50

^a – numbers at the end of the primer name refer to their relative position on the *mar* gene from strain *Rhodococcus opacus* B4; ^b - sequences are of the sense strand.

Appendix Table 0.4: Number of 454 reads for *pnpA* and *mar* gene.

Sample	<i>pnpA</i>		<i>mar</i>	
	Initial number of reads	Reads used for analysis	Initial number of reads	Reads used for analysis
T01	191	43	22	22
T02	10245	8687	27	19
T03	95	57	1907	978
HD1	27145	20497	763	713
HD2	12805	10715	6111	5755
HD3	14978	12327	13484	12776
Total	65459	52326	22314	20263

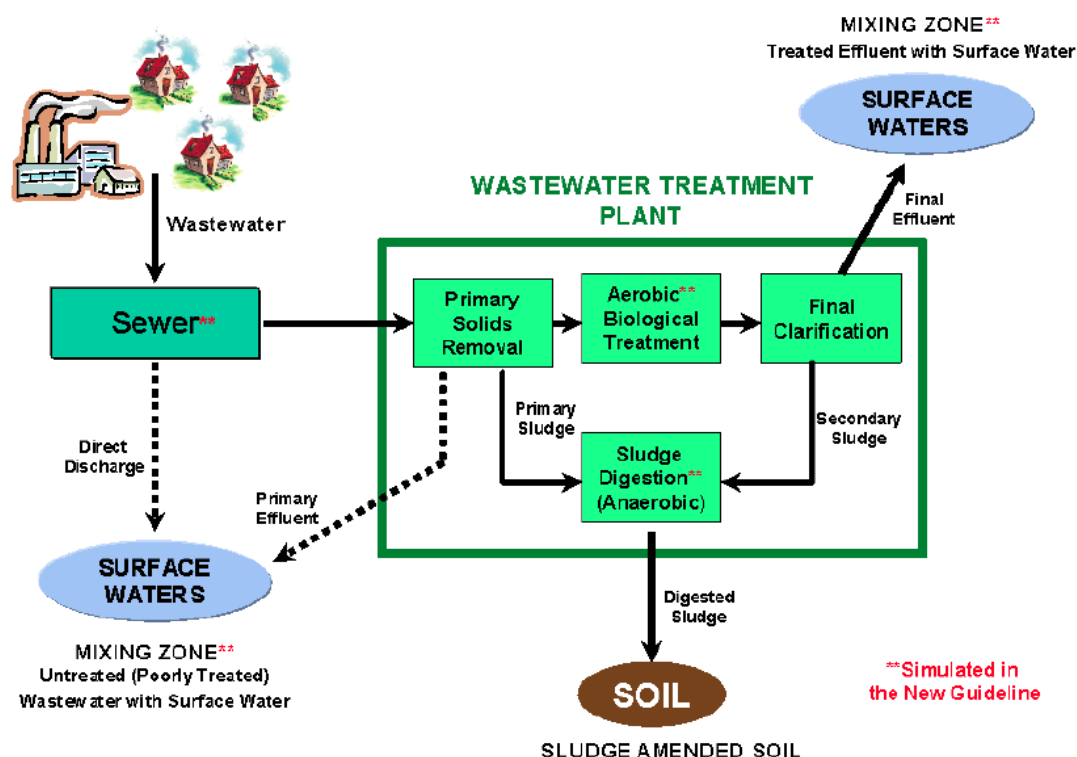
Appendix Table 0.5. Variation in bacterial communities based on Index of Multivariate Dispersion (IMD).

T₀	IMD	T_{end}	IMD	T₀ vs. T_{end}	IMD
D/E	-0.111	D/E	1	DT ₀ /DT _{end}	-0.111
D/SD	0.333	D/SD	0.333	DT ₀ /ET _{end}	0.333
D/SU	0.333	D/SU	0.556	DT ₀ /SDT _{end}	0.333
D/U	0.556	D/U	0.111	DT ₀ /SUT _{end}	0.333
E/SD	0.778	E/SD	-0.556	DT ₀ /UT _{end}	0.111
E/SU	0.778	E/SU	-0.333	DT _{end} /ET ₀	0.111
E/U	1	E/U	-1	DT _{end} /SDT ₀	0.778
SD/SU	0.111	SD/SU	0.556	DT _{end} /SUT ₀	0.778
SD/U	0.556	SD/U	-0.111	DT _{end} /UT ₀	1
SU/U	0.556	SU/U	0.556	ET ₀ /ET _{end}	1
				ET ₀ /SDT _{end}	0.333
				ET ₀ /SUT _{end}	0.556
				ET ₀ /UT _{end}	0.111
				ET _{end} /SDT ₀	-0.556
				ET _{end} /SUT ₀	-0.333
				ET _{end} /UT ₀	-0.111
				SDT ₀ /SDT _{end}	-0.556
				SDT ₀ /SUT _{end}	-0.111
				SDT ₀ /UT _{end}	-0.778
				SDT _{end} /SUT ₀	0.556
				SDT _{end} /UT ₀	0.556
				SUT ₀ /SUT _{end}	-0.556
				SUT ₀ /UT _{end}	-1
				SUT _{end} /UT ₀	-0.556
				UT ₀ /UT _{end}	-1

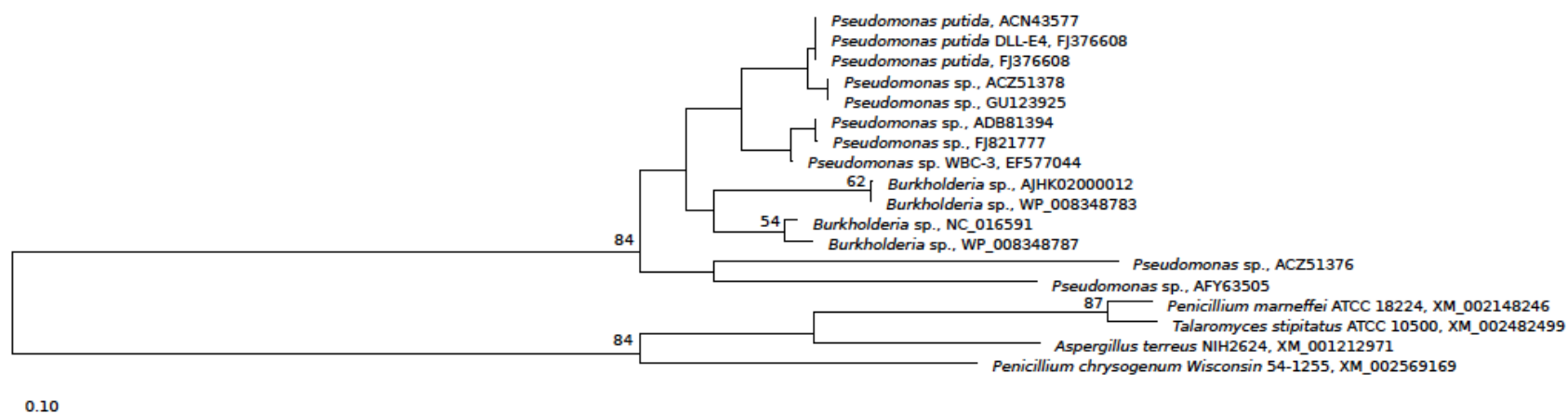
Where: **U**-river water upstream, **SU**-sediment extracts upstream, **E**- effluent river water, **D**-river water downstream, **SD**-sediment extract downstream; **T₀**-freshly collected river water and sediment extracts, **T_{end}**- river water and sediment extracts after PNP biodegradation; values close to “1” indicate high differences between treatments (extreme dispersion is when IMD=1), while values close to “0” indicate no difference between treatments (Warwick and Clarke, 1993).

Appendix Table 0.6: Slopes of rank-abundance plots for biofilm metacommunities.

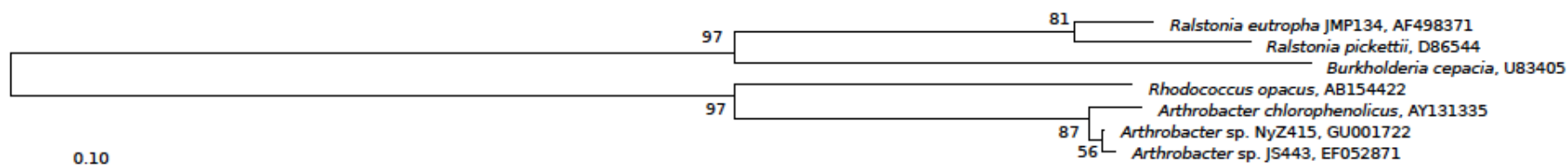
Metacommunity	Slope (<i>b</i>)	<i>r</i>²
November T ₀	-0.0632	0.94
November T _{end}	-0.0590	0.97
February T ₀	-0.0615	0.93
February T _{end}	-0.0402	0.98
May T ₀	-0.0242	0.97
May T _{end}	-0.0281	0.94



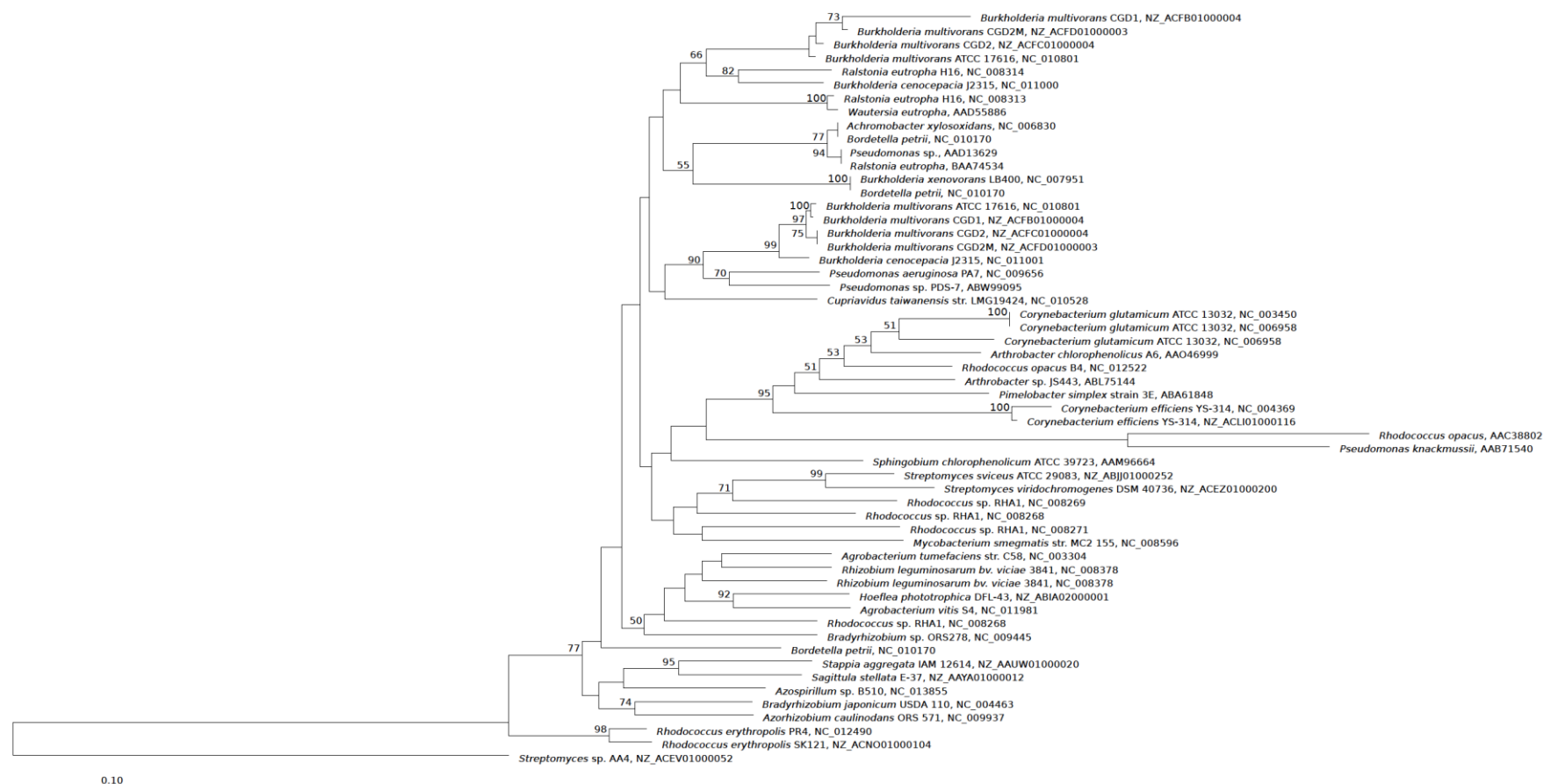
Appendix Figure 0.1: Diagram of OECD 314 with the most common transport pathways for chemicals discharged to wastewater. Source: OECD Guidelines for testing of chemicals, Test No. 314, 2008.



Appendix Figure 0.2: Neighbour-joining tree showing *pnpA* gene database used for primer design. Tree created based on nucleotide sequences translated into amino acid sequences using ARB (Ludwig *et al.*, 2004). Species name followed by *pnpA* sequence accession number are given. The scale bar represents 10 % sequence divergence. Numbers on each branch show the bootstrap value of 100 replicates (expressed as %). Branches with no values have less than 50 % bootstrap support.

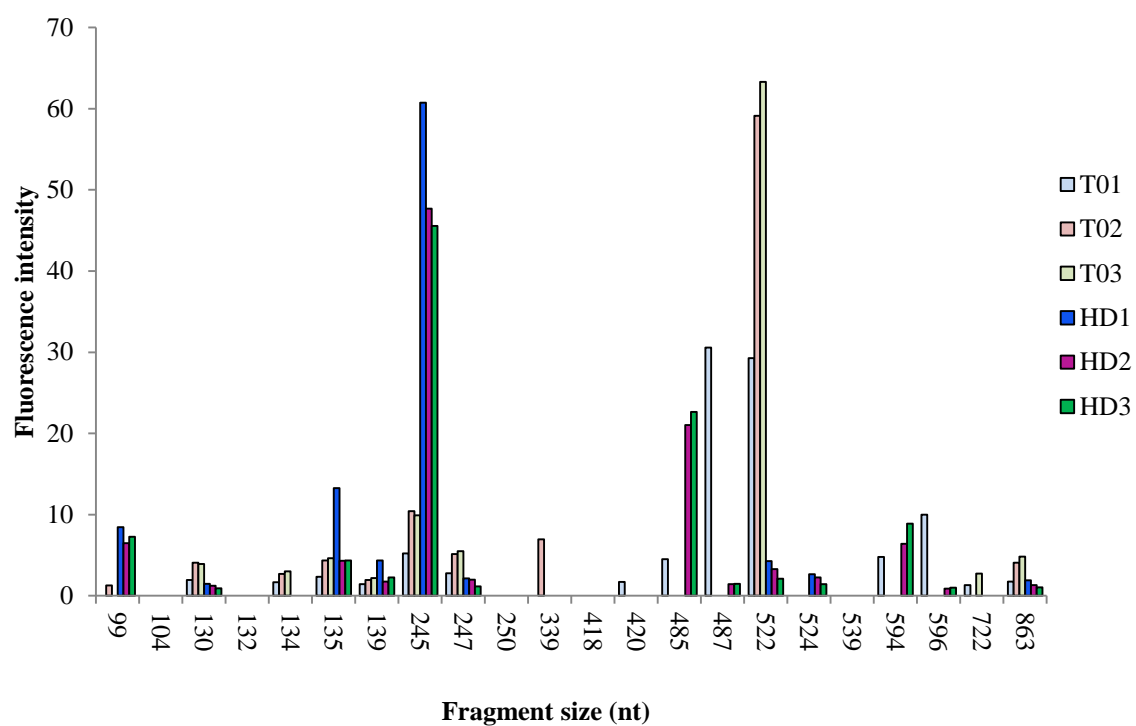


Appendix Figure 0.3: Neighbour-joining tree showing *npdA2* gene database used for primer design. Tree created based on nucleotide sequences translated into amino acid sequences using ARB (Ludwig *et al.*, 2004). Species name followed by *npdA2* sequence accession number are given. The scale bar represents 10 % sequence divergence. Numbers on each branch show the bootstrap value of 100 replicates (expressed as %). Branches with no values have less than 50 % bootstrap support.

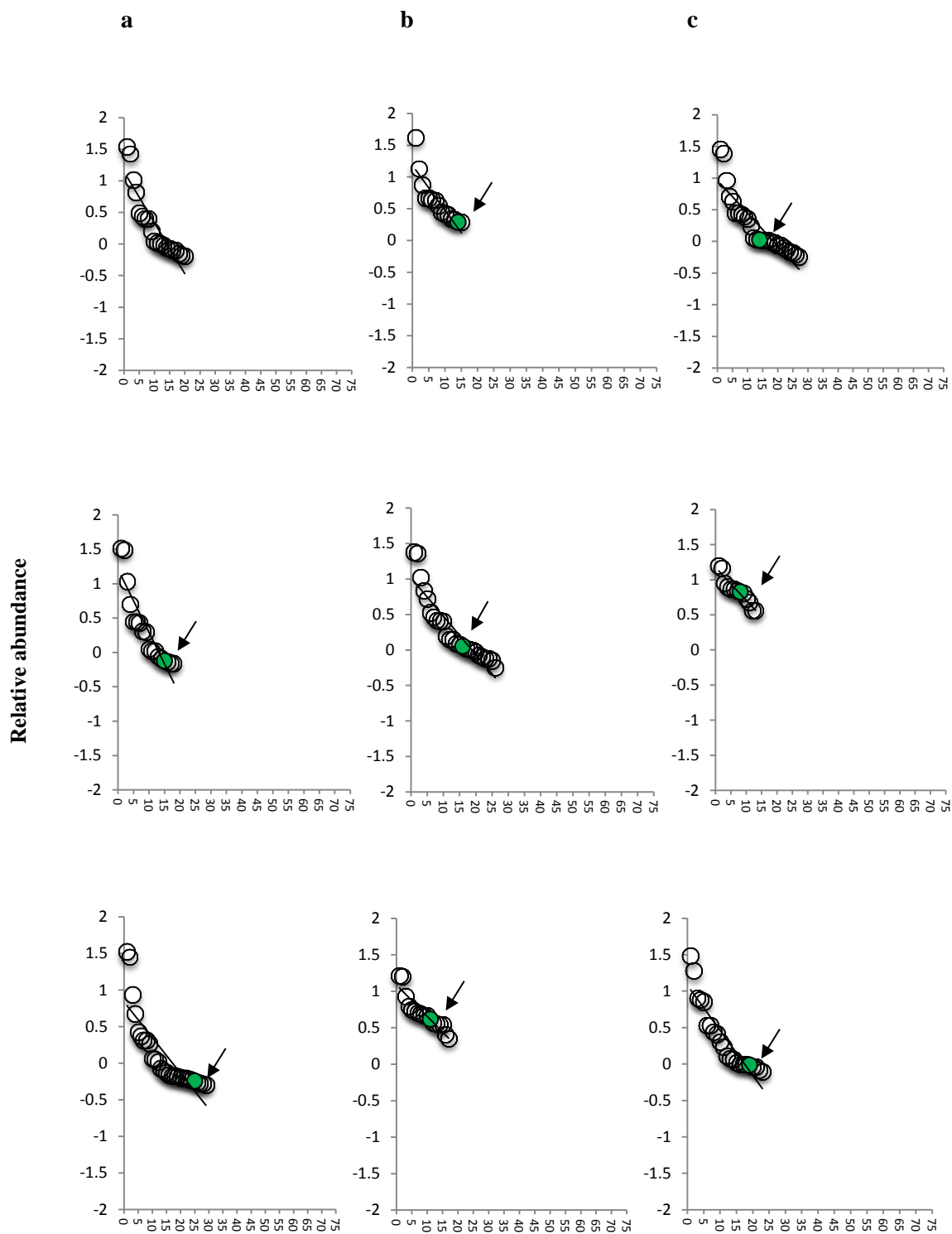


Appendix Figure 0.4: Neighbour-joining tree showing *mar* gene database used for primer design.

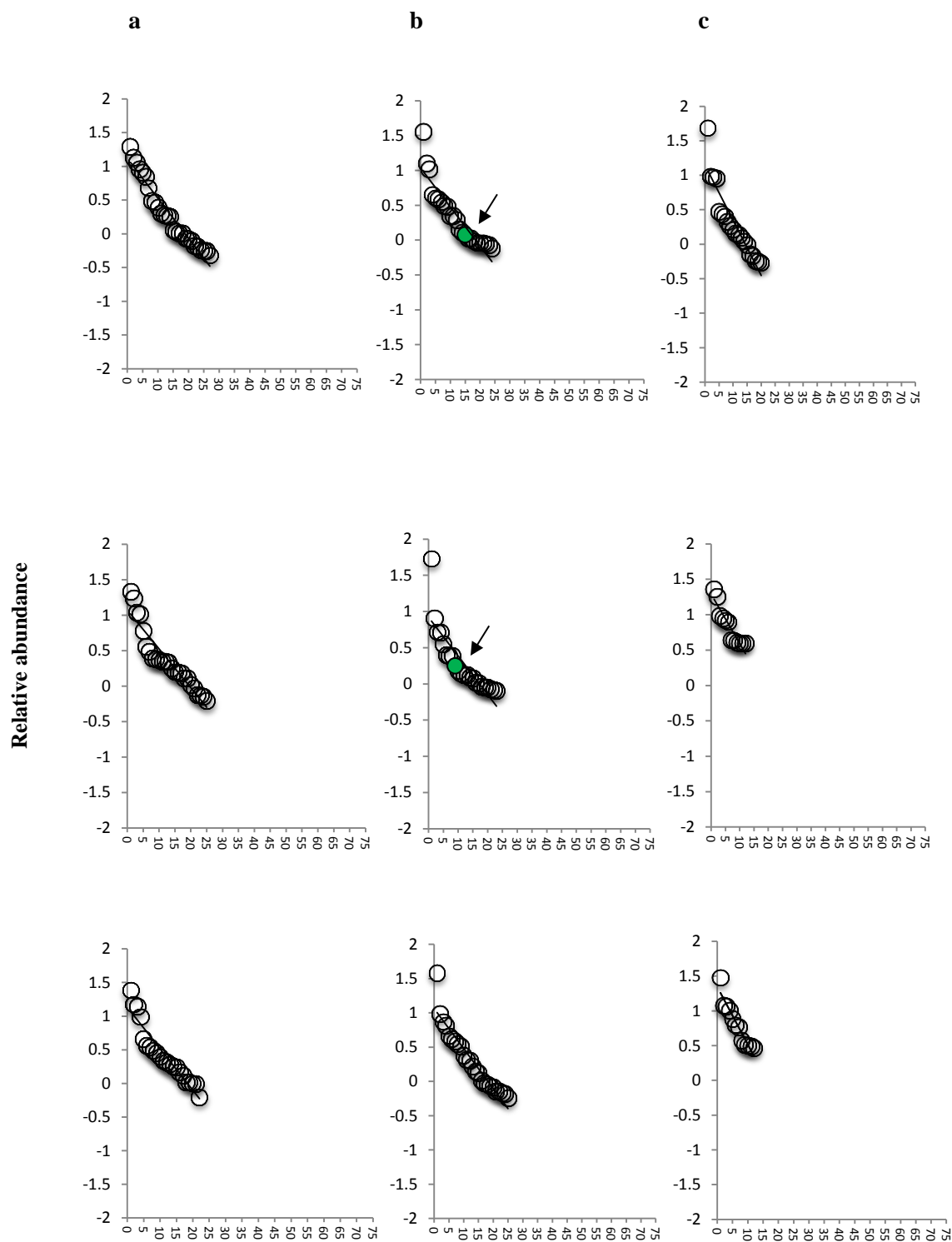
Tree created based on *mar* nucleotide sequences imported from IMG database and translated into amino acid sequences using ARB (Ludwig *et al.*, 2004). Species name followed by *mar* sequence accession number are given. The scale bar represents 10 % sequence divergence. Numbers on each branch show the bootstrap value of 100 replicates (expressed as %). Branches with no values have less than 50 % bootstrap support.



Appendix Figure 0.5: Example of bacterial 16S rRNA gene TRFLP profiles. Where T01-T03- replicates of freshly collected river water, and HD1-HD3- replicates of PNP degrading river water in the dark, TRF 245- previously identified as *Pseudomonas syringae*.

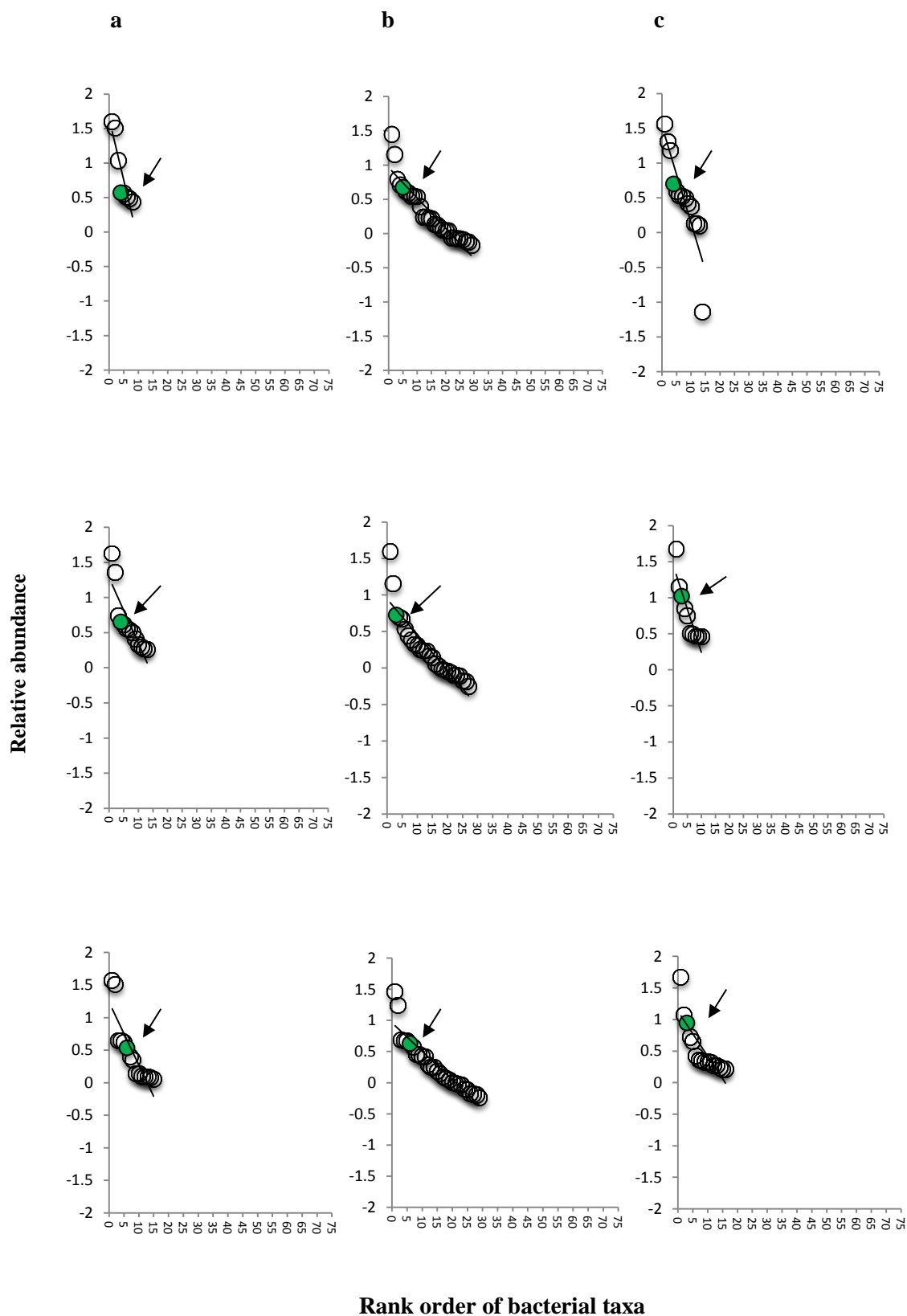


Rank order of bacterial taxa
Appendix Figure 0.6: Rank-abundance plots for local biofilm communities in November 2011 at T₀. The biofilm communities in replicate (n=3) from **a**- upstream, **b**- effluent and **c**- downstream sampling point. PNP-degrader, *Pseudomonas syringae* is highlighted in green and pointed out with an arrow.

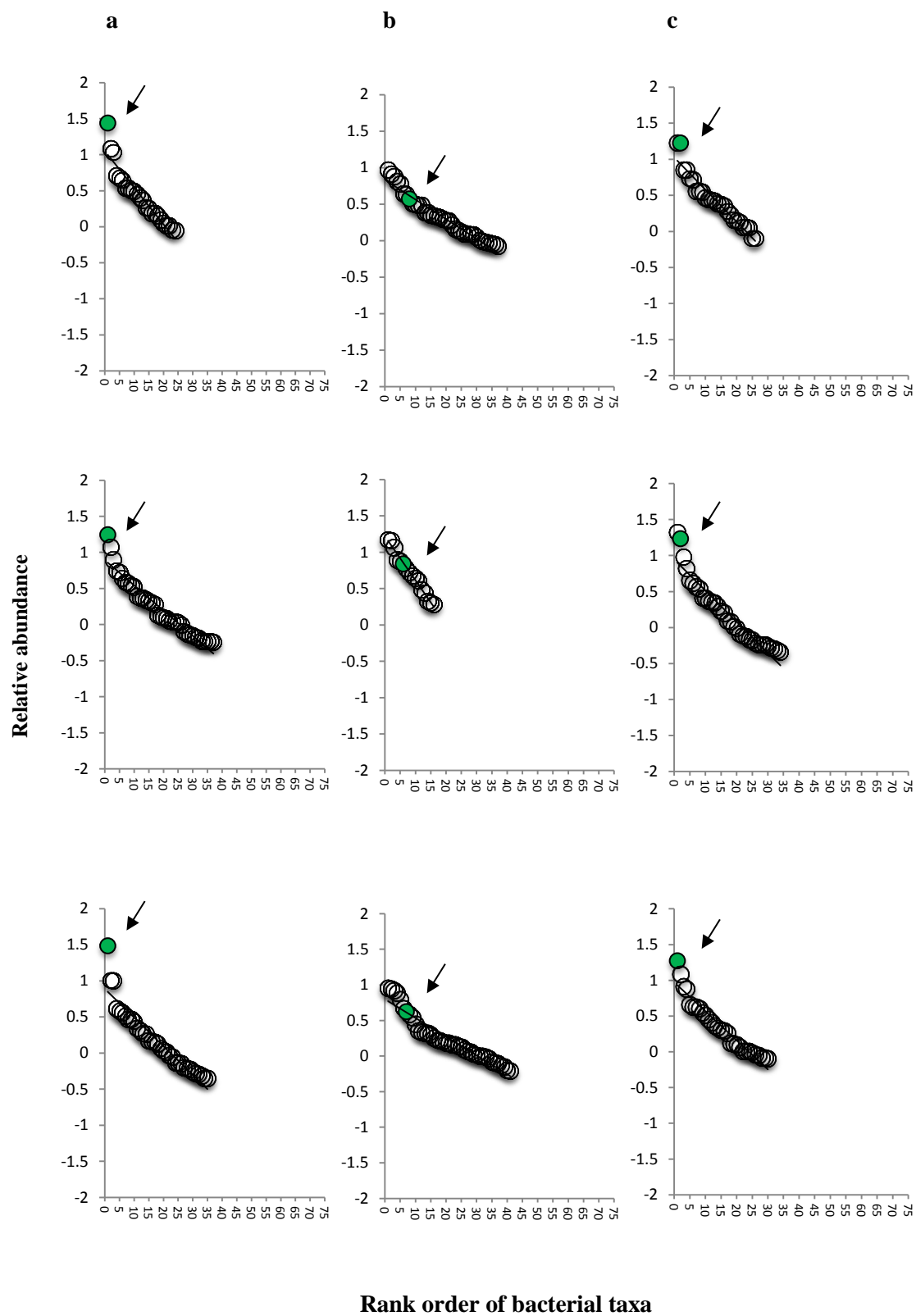


Rank order of bacterial taxa

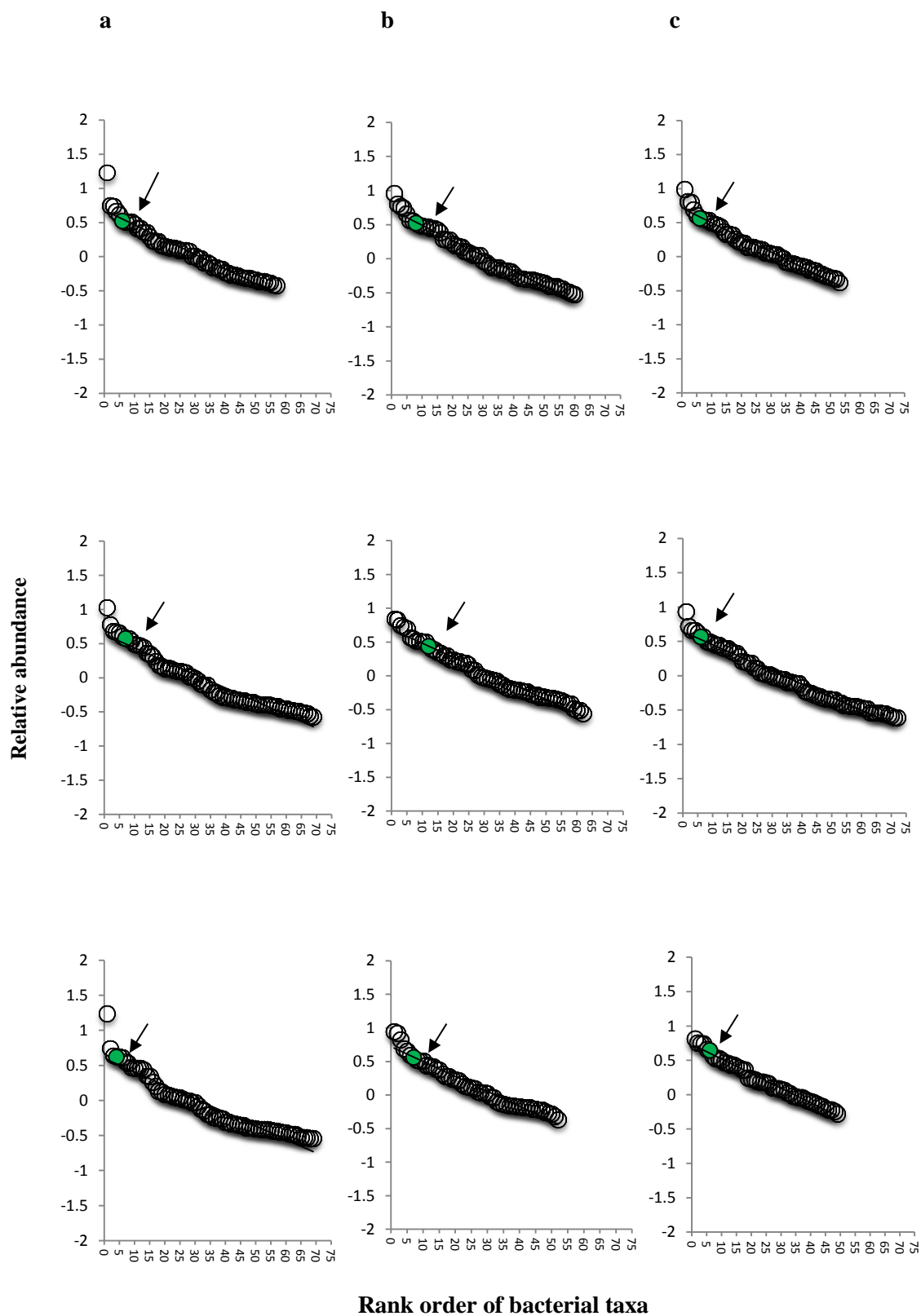
Appendix Figure 0.7: Rank-abundance plots for local biofilm communities in November 2011 at T_{end} . The biofilm communities in replicate (n=3) from **a**- upstream, **b**- effluent and **c**- downstream sampling point. PNP-degrader, *Pseudomonas syringae* is highlighted in green and pointed out with an arrow.



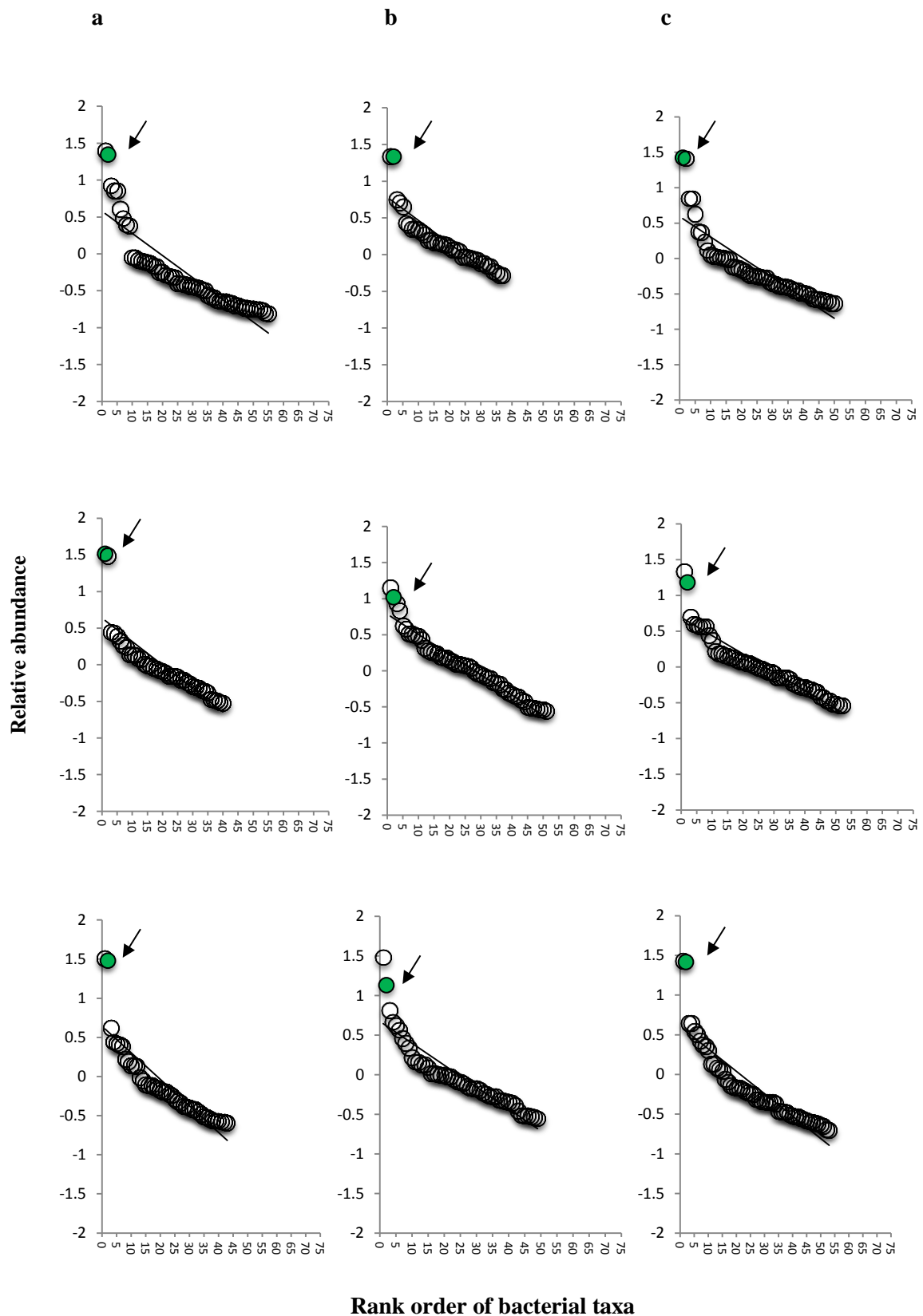
Appendix Figure 0.8: Rank-abundance plots for local biofilm communities in February 2012 at T₀. The biofilm communities in replicate (n=3) from **a**- upstream, **b**- effluent and **c**- downstream sampling point. PNP-degrader, *Pseudomonas syringae* is highlighted in green and pointed out with an arrow.



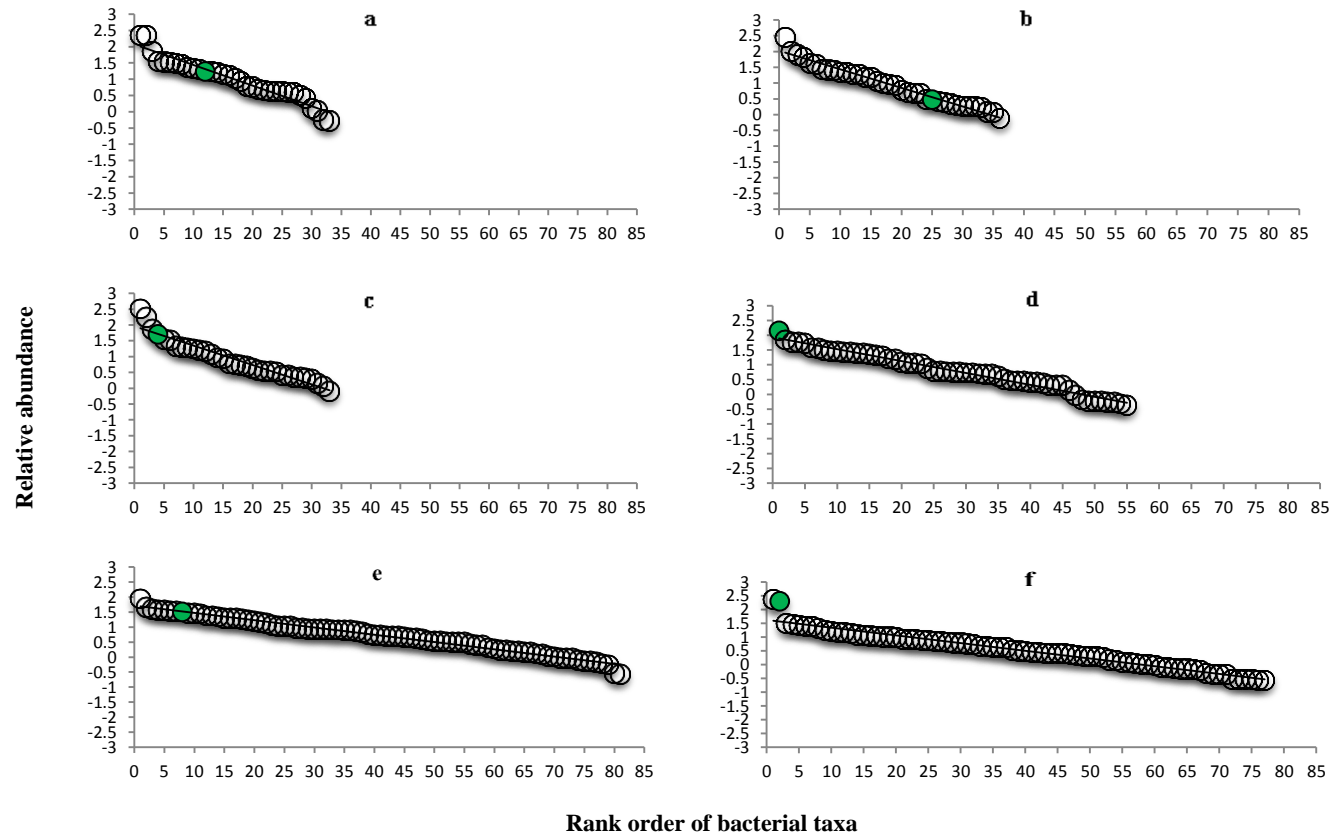
Appendix Figure 0.9: Rank-abundance plots for local biofilm communities in February 2012 at T_{end} . The biofilm communities in replicate ($n=3$) from **a**- upstream, **b**- effluent and **c**- downstream sampling point. PNP-degrader, *Pseudomonas syringae* is highlighted in green and pointed out with an arrow.



Appendix Figure 0.10: Rank-abundance plots for local biofilm communities in May 2012 at T_0 .
The biofilm communities in replicate ($n=3$) from **a**- upstream, **b**- effluent and **c**- downstream sampling point. PNP-degrader, *Pseudomonas syringae* is highlighted in green and pointed out with an arrow.

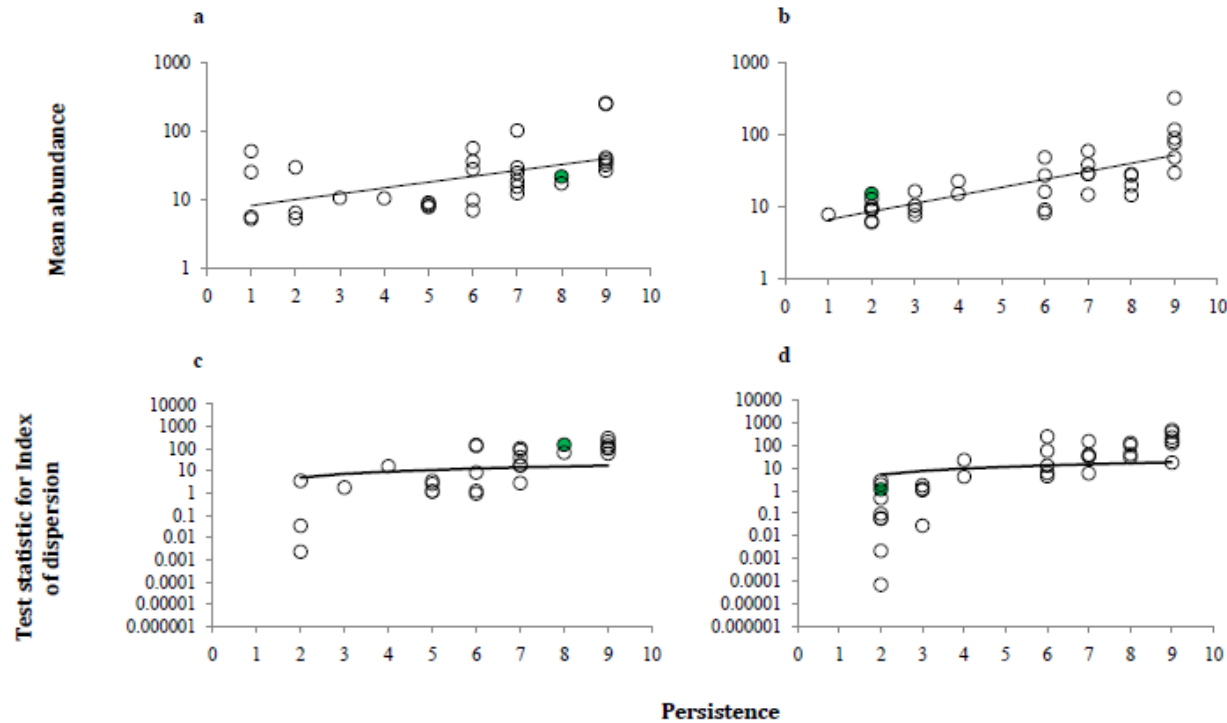


Appendix Figure 0.11: Rank-abundance plots for local biofilm communities in May 2012 at T_{end} . The biofilm communities in replicate (n=3) from **a**- upstream, **b**- effluent and **c**- downstream sampling point. PNP-degrader, *Pseudomonas syringae* is highlighted in green and pointed out with an arrow.



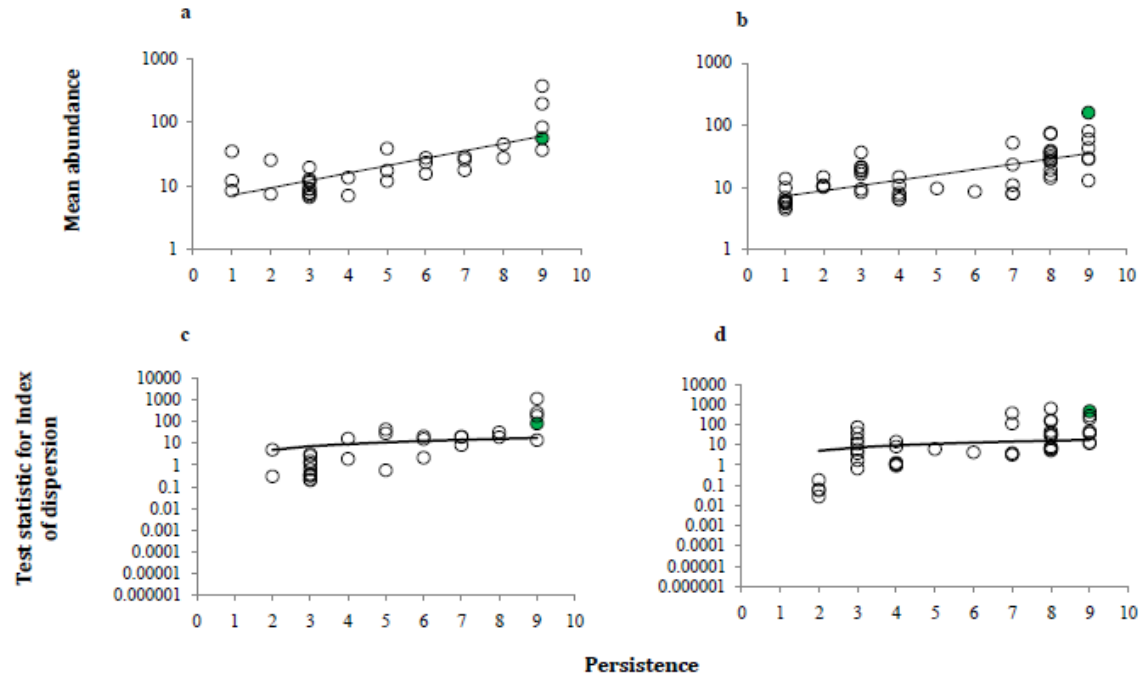
Appendix Figure 0.12: Rank-abundance plots of meatcommunities.

Where: **a, b** –November T_0 and T_{end} , **c, d**- February T_0 and T_{end} , **e, f**- May T_0 and T_{end} , where T_0 - freshly collected biofilms, T_{end} – biofilms after complete PNP biodegradation and *Pseudomonas syringae* is highlighted in green. Each plot has been fitted with a slope.



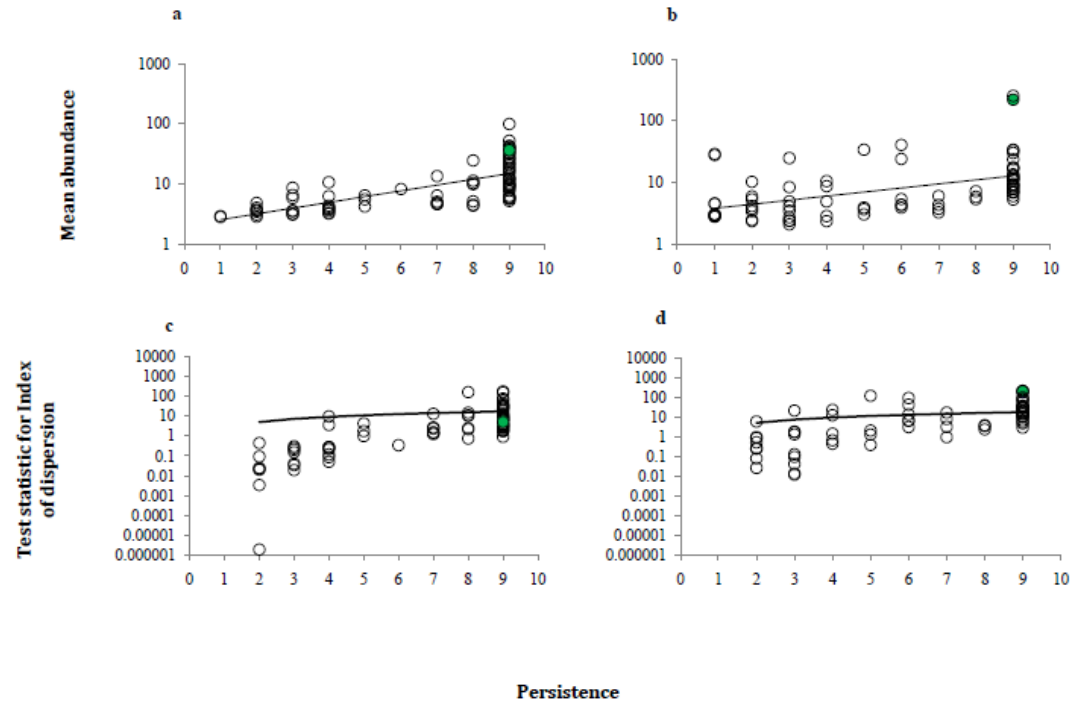
Appendix Figure 0.13: Distribution and dispersal of bacterial taxa across TRFLP profiles from November biofilms.

The number of biofilm TRFLP profiles for which each bacterial TRF (taxa) was observed, plotted against the mean abundance (log₁₀ scale) across all TRFLP profiles **a**- before incubation (T_0) ($r^2 = 0.29$; $F_{1,32} = 12.57$; $P < 0.0001$), and **b**- after complete PNP biodegradation (T_{end}) ($r^2 = 0.60$; $F_{1,35} = 51.57$; $P < 0.0001$). **c**, **d**- random and non-random dispersal of TRFs from biofilm TRFLP profiles (*P. syringae* highlighted in green). Dispersal visualised by decomposing the overall distribution using an index of dispersion based on the ratio of variance to the mean abundance for each bacterial TRF from analysed 9 TRFLP profiles. The line depicts the 2.5% confidence limit for the χ^2 distribution. The 97.5% confidence limit was not plotted, as no taxon fell below that line.



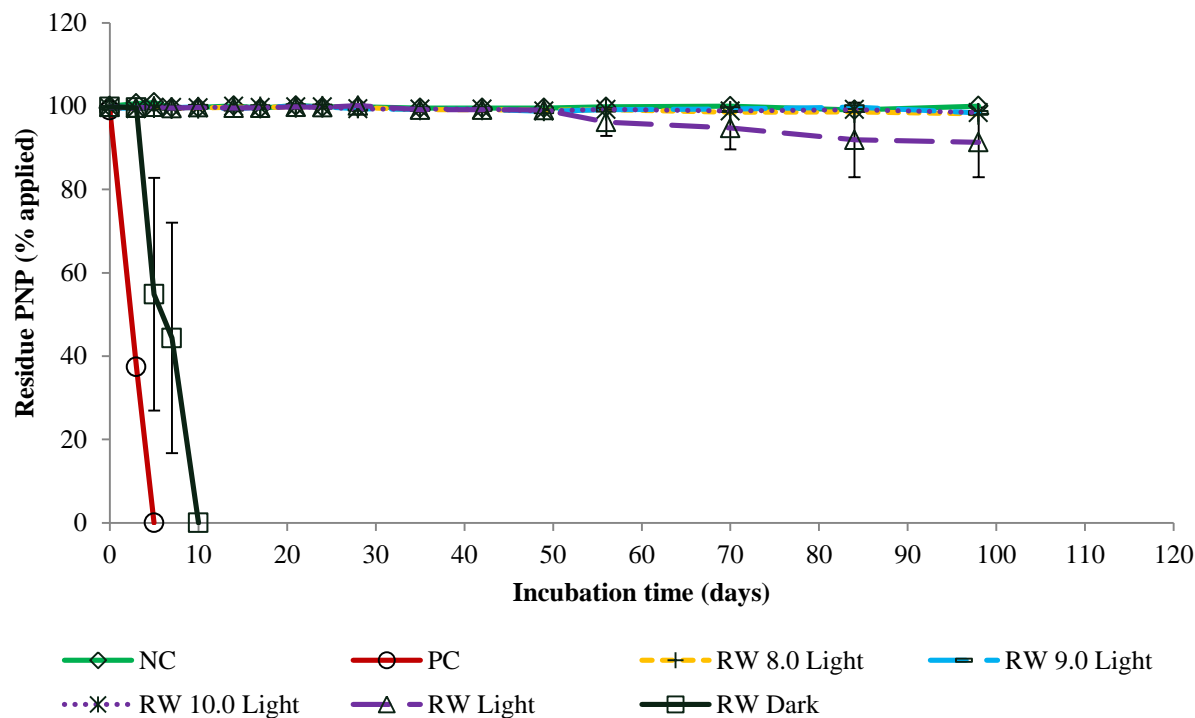
Appendix Figure 0.14: Distribution and dispersal of bacterial taxa across TRFLP profiles from February biofilms.

The number of biofilm TRFLP profiles for which each bacterial TRF (taxa) was observed, plotted against the mean abundance (log₁₀ scale) across all TRFLP profiles **a**- before incubation (T_0) ($r^2 = 0.55$; $F_{1,32} = 37.52$; $P < 0.0001$), and **b**- after complete PNP biodegradation (T_{end}) ($r^2 = 0.51$; $F_{1,54} = 54.91$; $P < 0.0001$). **c**, **d**- random and non-random dispersal of TRFs from biofilm TRFLP profiles (*P. syringae* highlighted in green). Dispersal visualised by decomposing the overall distribution using an index of dispersion based on the ratio of variance to the mean abundance for each bacterial TRF from analysed 9 TRFLP profiles. The line depicts the 2.5% confidence limit for the χ^2 distribution. The 97.5% confidence limit was not plotted, as no taxon fell below that line.

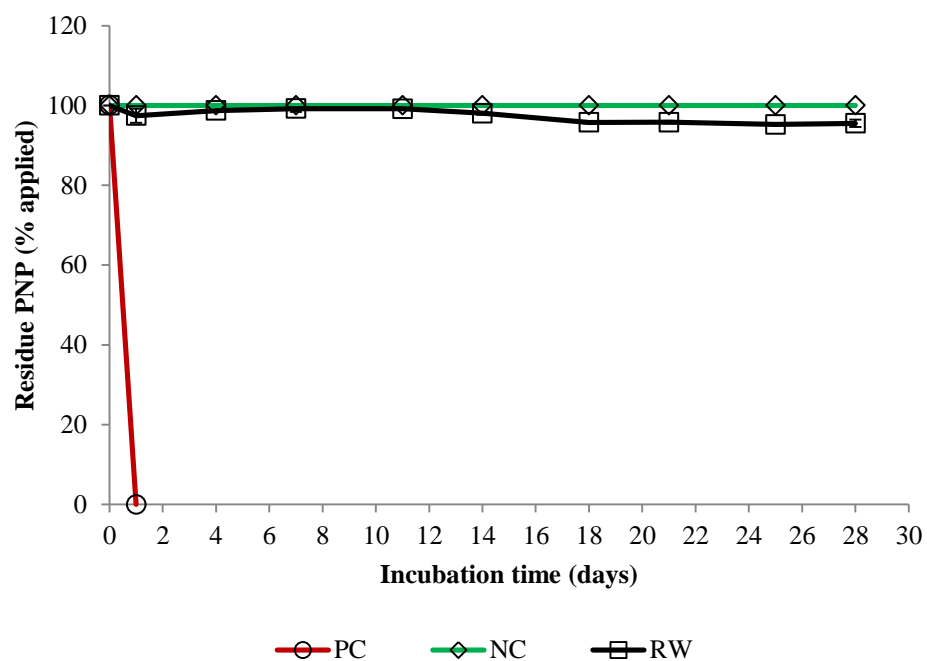


Appendix Figure 0.15: Distribution and dispersal of bacterial taxa across TRFLP profiles from May biofilms.

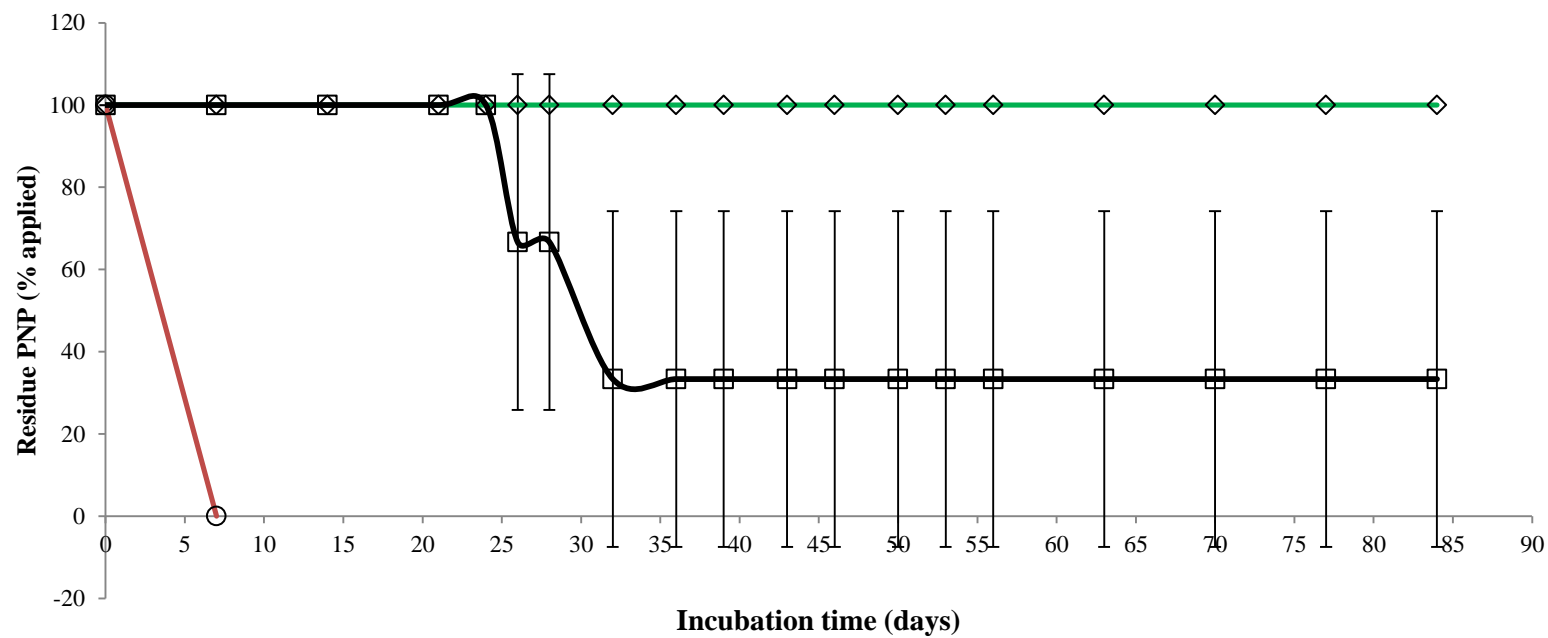
The number of biofilm TRFLP profiles for which each bacterial TRF (taxa) was observed, plotted against the mean abundance (\log_{10} scale) across all TRFLP profiles **a**- before incubation (T_0) ($r^2 = 0.50$; $F_{1,80}=78.10$; $P<0.0001$), and **b**- after complete PNP biodegradation (T_{end}) ($r^2 = 0.23$; $F_{1,76}=22.04$; $P<0.0001$). **c**, **d**- random and non-random dispersal of TRFs from biofilm TRFLP profiles (*P. syringae* highlighted in green). Dispersal visualised by decomposing the overall distribution using an index of dispersion based on the ratio of variance to the mean abundance for each bacterial TRF from analysed 9 TRFLP profiles. The line depicts the 2.5% confidence limit for the χ^2 distribution. The 97.5% confidence limit was not plotted, as no taxon fell below that line.



Appendix Figure 0.16: The biodegradation of PNP at concentration of 2mg/L in the dark incubated river water and light incubated river water with variable pH (July 2011). Figure legend: NC- negative control; PC- positive control; RW – river water with native pH=7.8, **RW 8.0** – river water with pH adjusted to 8.0; **RW 9.0** – river water with pH adjusted to 9.0, **RW 10.0** – river water with pH adjusted to 10.0, **Light** – light incubation, **Dark**- dark incubation; error bars showing standard error of the mean (S.E.M.), n=3.



Appendix Figure 0.17: ^{14}C PNP biodegradation at PNP concentration of 2mg/L with river water inoculum (July 2011). Figure legend: **PC** – positive control, **NC** – negative control, **RW** – river water; error bars showing standard error of the mean (S.E.M.), $n=3$.



Appendix Figure 0.18: ^{14}C PNP biodegradation at PNP concentration of 2mg/L with river water inoculum (January 2012).
 Figure legend: NC – negative control; PC – positive control; RW – river water; error bars showing standard error of the mean (S.E.M.), n=3.

